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(54) Title: **PARAMYCOBACTERIAL DIAGNOSTICS AND VACCINES**

(57) Abstract: The present invention relates to nucleic acid sequences encoding Mycobacterium avium subspecies paratuberculosis proteins, to parts of such nucleic acid sequences that encode an immunogenic fragment of such proteins, to DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof. The invention also relates to Mycobacterium avium subspecies paratuberculosis proteins and immunogenic parts thereof encoded by such sequences. Furthermore, the present invention relates to vaccines comprising such nucleic acid sequences and parts thereof, DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof, proteins or immunogenic parts thereof and antibodies against such proteins or immunogenic parts thereof. Also, the invention relates to the use of said proteins in vaccines and for the manufacture of vaccines. Moreover, the invention relates to the use of said nucleic acid sequences, proteins or antibodies for diagnostic or vaccination purposes. Also, the invention relates to methods for the preparation of such vaccines. Finally the invention relates to diagnostic kits comprising such nucleic acids, proteins or antibodies against such proteins.

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Paramycobacterial diagnostics and vaccines.

The present invention relates to nucleic acid sequences encoding *Mycobacterium avium* subspecies *paratuberculosis* proteins, to parts of such nucleic acid sequences that encode an immunogenic fragment of such proteins, to DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof. The invention also relates to *Mycobacterium avium* subspecies *paratuberculosis* proteins and immunogenic parts thereof encoded by such sequences. Furthermore, the present invention relates to vaccines comprising such nucleic acid sequences and parts thereof, DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof, proteins or immunogenic parts thereof and antibodies against such proteins or immunogenic parts thereof. Also, the invention relates to the use of said proteins in vaccines and for the manufacture of vaccines. Moreover, the invention relates to the use of said nucleic acid sequences, proteins or antibodies for diagnostic or vaccination purposes. Also, the invention relates to methods for the preparation of such vaccines. Finally the invention relates to diagnostic kits comprising such nucleic acids, proteins or antibodies against such proteins.

Bacteria of the genus *Mycobacterium* are Gram-positive acid-fast organisms. The genus includes a number of significant human and animal pathogens. Amongst these is *M. avium* subspecies *paratuberculosis*, the causative agent of paratuberculosis or Johne's disease, a chronic granulomatous infection leading to disease in ruminants that is currently responsible for very substantial worldwide economic losses in both meat and dairy industry. A large proportion of herds (between 21-70%) are infected worldwide. In Europe, the estimated yearly losses are about GBP 207/diary cow. In the USA, the estimated yearly losses are about 1.5 billion US-dollars. (Harris, N.B. and Barletta, R.G., Clinical Microbiology Reviews 14: 489-512 (2001)).

In addition to its evident pathogenicity in ruminants, *M. avium* subspecies *paratuberculosis* is suspected to be the cause of Crohn's disease, a non-specific chronic transmural inflammatory disease of humans that most commonly affects the distal ileum and colon but that may also occur in any part of the

gastrointestinal tract from the mouth to the anus and perianal area. (P. Quirke, Gut 2001, 49: 755-760 (2001)), ("Possible links between Crohn's disease and Paratuberculosis", European Commission, D-G Health and Consumer Protection, Report of the Scientific Committee on Animal Health and Animal Welfare, 21 March 2000). One of the main problems of Crohn's disease is the fact that there is no cure for the disease. The disease status, once gathered, remains present life-long, causing significant morbidity.

The presence of unexpectedly more thermally tolerant strains of *M. avium* subspecies *paratuberculosis* in pasteurised milk in combination with its suspected role in the development of Crohn's disease has raised increasing concern regarding its potential health effects on the human population as well. This recent and unexpected finding has been described by Sung, N. and Collins, M.T. in Appl. Environm. Microbiol. 64: 999-1005 (1998) .

Increased awareness of the problem has resulted in renewed urgency for the development of effective diagnostics and vaccines for control and eradication of paratuberculosis.

*Mycobacterium avium* comprises a large group of mycobacteria that can be divided into three subspecies, *M. avium* subspecies *avium*, *M. avium* subspecies *silvaticum* and *M. avium* subspecies *paratuberculosis*. *M. avium* subspecies *avium* is widely distributed in the natural environment including soil and apparently healthy animals, as well as in birds and humans. *M. avium* subspecies *avium* isolates are opportunistic pathogens and generally cause infection and disease in immunocompromised hosts. The complete genomic sequence of *M. avium* subspecies *avium* strain 104 is currently being determined. *M. avium* subspecies *silvaticum* can produce a disease that resembles paratuberculosis in deer. Although most ruminants are infected with *M. avium* subspecies *paratuberculosis* before six months of age, clinical disease generally occurs only after at least two years of age, or later. During this period, bacteria are believed to survive inside host cells, but extracellular episodes of infection in the lumen of the gastrointestinal tract -during which the bacterium becomes detectable in faeces -do also occur (with increasing frequency at later stages of infection). Currently available (immuno-) diagnostics against *M. avium* subspecies *paratuberculosis* have a relatively poor sensitivity,

especially with respect to the detection of early or latent infection, and therefore are not effective as a tool for disease control. Whole cell Mycobacterial vaccines that are to some measure thought to be effective in freeing herds from clinical disease are available, but these vaccines essentially interfere with the immunodiagnosis of bovine tuberculosis and do not inhibit transmission of disease. To date several antigenic components of *M. avium* subspecies *paratuberculosis* have been identified. The antigenic molecules of *M. avium* subspecies *paratuberculosis* described previously comprise glycolipids and protein antigens identified with essentially monospecific early sera raised in small experimental animals. The cell wall glycolipid molecule lipoarabinomannan (LAM) was identified by its recognition by monoclonal antibodies raised against cell filtrate released by the bacterium, and has subsequently been purified and used for the development of a serodiagnostic ELISA (Mutharia et al., Infect. Immun. 1997.65:387-394; Jark et al., 1997. Vet. Microbiol. 57:189-198). In addition, protein antigens with molecular weight of 14 kD (Olsen et al. Clin. Diagn. Lab. Immunol. 2001.8:797-801), 18 kD (bacterioferritin; Elsaghier et al. Clin. Exp. Immunol. 1992 90:503-508), 19 kD (AhpD; Olsen et al., Infect. Immun. 2000.68:801-808), 24 kD (p24BCD; Elsaghier et al. Clin. Exp. Immunol. 1992 90:503-508), 30 kD (p30; Burrels et al.; Vet. Immunol. Immunopathol. 1995. 45:311-320), 34 kD (Gilot et al. J. Bact. 1993. 175:4930-4935; De Kesel et al J. Clin. Microbiol. 1993. 31: 947-954; Coetsier et al., Clin. Diagn. Lab. Immunol. 1998.5: 446-451), 34.5 kD (Mutharia et al., Infect. Immun. 1997.65:387-394), ), a 35 kD protein (Dheenadhayalan and Chang, unpublished data), 38 kD (Elsaghier et al. Clin. Exp. Immunol. 1992.90:503-508), 44.3 kD (Mutharia et al., Infect. Immun. 1997.65:387-394), 45 kD (AhpC; Olsen et al., Infect. Immun. 2000. 68:801-808), 65 kD (hsp65; Koets et al., Vet. Immunol. Immunopathol. 1999. 70:105-115), 70 kD (hsp70; Stevenson et al., 1991. Nucleic Acids Res. 19:4552; Koets et al., Vet. Immunol. Immunopathol. 1999.70:105-115), and a superoxide dismutase molecule (Mullerad et al., FEMS Immunol. Med. Microbiol 34: 81 (2002)) have been identified and (partly) characterized. Only few of these have been evaluated for the development of diagnostics or vaccines (34 kD; Coetsier et al., Clin. Diagn. Lab. Immunol. 1998. 5: 446-451). Current diagnostics and vaccines are therefore still based on rather crude antigenic materials. The lipoarabinomannan (Mutharia et al., Infect. Immun. 1997. 65:387 - 394; Jark et al., 1997. Vet. Microbiol. 57:189-198) and 34 kD antigen (Gilot et al. J. Bact.

1993. 175:4930-4935; De Kesel et al J. Clin. Microbiol. 1993. 31: 947- 954; Coetsier et al., Clin. Diagn. Lab. Immunol. 1998.5: 446-451) have been described in DE19621488 and WO9216628, for use in diagnosis and vaccines. Several other molecules have been submitted for use in diagnostics, vaccines and therapeutics.

5 These are proteins encoded on insertion sequence ISM-1 (EPO288306 and US 5225324;), the mycobacterial DAP molecule (US9523226), a 36 kD antigen (US5776692), a soluble antigen preparation (RU2118538), an extra cellular protein with an iron-reducing capacity (DE 19728834), and an acylase (WO9949054).

10

It is an objective of the present invention to provide polypeptides that are capable of contributing to protection against the pathogenic effects of *Mycobacterium avium* subspecies *paratuberculosis* infection in mammals, more specifically humans and cattle. Moreover, a number of these polypeptides and antibodies against these

15 polypeptides provide efficient diagnostic tools.

It was now first of all surprisingly found that nine different polypeptides could be specifically identified in expression libraries and isolated, and two additional polypeptides could be identified in proteomics, each of these different polypeptides

20 being capable of inducing an immune response against *Mycobacterium avium* subspecies *paratuberculosis* and suitable as vaccine components.

The inventors have found that these polypeptides can be used, either alone or in combination with each other, as vaccine components to provide a vaccine which indeed contributes to the protection against *Mycobacterium avium* subspecies

25 *paratuberculosis* infection in mammals, more specifically in humans and cattle and helps to decrease the damage caused by *Mycobacterium avium* subspecies *paratuberculosis*.

Three different approaches have been used for the detection of the (genes encoding

30 the) vaccine components and diagnostic tools according to the invention. These approaches are presented in more detail in the Examples. One approach uses a very specific antiserum for the detection of genes encoding immunoreactive *Mycobacterium avium* subspecies *paratuberculosis* proteins in an expression library. The antiserum used differs from antisera commonly used for the screening

of expression libraries in the sense that is has been obtained from cows that have been infected with *Mycobacterium avium* subspecies *paratuberculosis* for a very prolonged period. Furthermore, these antisera were taken from cows that were found to be naturally infected with *M. avium subsp. paratuberculosis*, but had no history of infection with tuberculosis, brucellosis or leucosis. This was evidenced by finding at least two *M. avium subsp. paratuberculosis* positive faeces samples within an approximately two year long period before obtaining the test-serum for use in screening, and finding essentially no antibodies or other immune responses directed against agents causing tuberculosis that are cross-reactive with paratuberculosis antigens. Sera thus obtained are very useful in immunoscreening for *M. avium subsp. paratuberculosis* antigens, since they are very broadly reactive against relevant *M. avium subsp. paratuberculosis* peptide fragments whereas on the other hand they show essentially no or only little specific reactivity with the pathogens causing tuberculosis, brucellosis or leucosis.

This approach has led to the finding of three novel immunogenic proteins for which the coding sequences are depicted in SEQ ID NO: 1, 3, and 5 as given below.

The gene encoding the first protein has now been cloned and sequenced and a nucleic acid sequence of the gene that comprises immunogenic determinants is depicted in SEQ ID NO: 1. The full-length gene encodes a protein (as depicted in SEQ ID NO: 2) with a molecular mass of 28 kD.

It is well-known in the art, that many different nucleic acid sequences can encode one and the same protein. This phenomenon is commonly known as wobble in the second and especially the third base of each triplet encoding an amino acid. This phenomenon can result in a heterology for two nucleic acid sequences still encoding the same protein. Therefore, in principle, two nucleic acid sequences having a sequence homology as low as 70 % can still encode one and the same protein.

Thus, one form of a first embodiment of the present invention relates to a nucleic acid sequence encoding an *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has

at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 1.

The concept of immunogenic fragment is defined below. The length of a nucleic acid sequence encoding an immunogenic fragment is usually at least 18 or more often 21 nucleotides, but preferably 24, 27, 30, 33 or even 36 nucleotides.

The molecular weight of all proteins according to the invention when determined in gel electrophoresis on a polyacryl amide gel may vary to a certain extend, due to slight variability of molecular weight determination frequently encountered in the art. Therefore the molecular weight of the proteins according to the invention should be interpreted as to be its theoretical molecular weight +/- 5 kD.

Preferably, a nucleic acid sequence according to the invention encoding this *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 1.

Even more preferred is a homology level of 98%, 99% or even 100%.

The level of nucleotide homology can be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTN" that can be found at [www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

A reference for this program is Tatiana A. Tatusova, Thomas L. Madden FEMS Microbiol. Letters 174: 247-250 (1999). Parameters used are the default parameters:

Reward for a match: +1. Penalty for a mismatch: -2. Open gap: 5. Extension gap: 2.

Gap x\_dropoff: 50.

Nucleotide sequences that are complementary to the sequence depicted in SEQ ID NO 1 or any of SEQ ID NO 3, 5, 7, 9, 11, 13, 15 or 17 that will be described below, or nucleotide sequences that comprise tandem arrays of the sequences according to the invention, are also within the scope of the invention.

5

Another form of this embodiment relates to a nucleic acid sequence encoding a 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 3.

10

Preferably, a nucleic acid sequence according to the invention encoding this 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 3.

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Even more preferred is a homology level of 98%, 99% or even 100%.

20

Still another form of this embodiment relates to a nucleic acid sequence encoding a 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 5.

25

Preferably, a nucleic acid sequence according to the invention encoding this 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 5.

30



Even more preferred is a homology level of 98%, 99% or even 100%.

5 Another approach used for the detection of immunologically important polypeptides was based upon the use of highly specific monoclonal antibodies against *Mycobacterium avium* subspecies *paratuberculosis* proteins. This approach has the advantage that it is even more specific than the approach using the specific antisera against *Mycobacterium avium* subspecies *paratuberculosis* described  
10 above.  
Use of these monoclonal antibodies led to the identification and isolation of six additional immunogenic *Mycobacterium avium* subspecies *paratuberculosis* proteins.

15 Therefore, again another form of this embodiment relates to a nucleic acid sequence encoding a 47 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium*  
20 subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 7.

Preferably, a nucleic acid sequence according to the invention encoding this 47 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least  
25 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 7.

Even more preferred is a homology level of 98%, 99% or even 100%.

30 Again another form of this embodiment relates to a nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology

with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 9.

Preferably, a nucleic acid sequence according to the invention encoding this  
5 *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 9.

10

Even more preferred is a homology level of 98%, 99% or even 100%.

Another form of this embodiment relates to a nucleic acid sequence encoding a  
15 *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 11.

20 Preferably, a nucleic acid sequence according to the invention encoding this *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as  
25 depicted in SEQ ID NO: 11.

Even more preferred is a homology level of 98%, 99% or even 100%.

Again another form of this embodiment relates to a nucleic acid sequence encoding  
30 a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 13.

Preferably, a nucleic acid sequence according to the invention encoding this *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 13.

Even more preferred is a homology level of 98%, 99% or even 100%.

Again another form of this embodiment relates to a nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 15.

Preferably, a nucleic acid sequence according to the invention encoding this *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 15.

Even more preferred is a homology level of 98%, 99% or even 100%.

Again another form of this embodiment relates to a nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein wherein said nucleic acid sequence or said part thereof has at least 85 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 17.

Preferably, a nucleic acid sequence according to the invention encoding this *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of that nucleic acid sequence that encodes an immunogenic fragment of that protein has at least 90 %, preferably 93 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 17.

Even more preferred is a homology level of 98%, 99% or even 100%.

A third approach, based upon careful analysis of the proteome of *Mycobacterium avium* subspecies *paratuberculosis* has led to the detection of again two novel vaccine components. This approach is based upon identification of immunogenic proteins in 2D-gels. It has the advantage over the other approaches, that proteins not yet found or identified in an expression library can now be unambiguously identified as vaccine components. Details of the methods followed are given in Example 2.

Thus, still another embodiment of the present invention relates to a 60 kD *Mycobacterium avium* subspecies *paratuberculosis* protein that has a pI between 5.60 and 6.15.

This protein is visible as a horizontal row of about 5 spots (due to small differences in isoforms representing e.g. different post-translational modifications or artefacts introduced by the preparation of the samples for 2D-gel electrophoresis) in figure 1 b and d.

Additionally, another embodiment relates to a 33 kD *Mycobacterium avium* subspecies *paratuberculosis* protein that has a pI between 4.20 and 4.75.

This protein is visible as a horizontal row of about 3 spots (again due to small differences in isoforms representing e.g. different post-translational modifications or artefacts introduced by the preparation of the samples for 2D-gel electrophoresis) in figure 1 a and d.

Since these proteins have now been unambiguously identified, they can be sequenced, e.g. the first 15 N-terminal amino acids can be determined according to standard procedures known in the art. Such N-terminal sequencing is nowadays e.g. commercially and on a routine basis done by companies specialised in protein  
5 sequencing. The genes encoding these proteins can then easily be identified using degenerate probes. These techniques are all well-known in the art.

Since the present invention discloses nucleic acid sequences encoding novel *Mycobacterium avium* subspecies *paratuberculosis* proteins, it is now for the first  
10 time possible to obtain these proteins in sufficient quantities. This can e.g. be done by using expression systems to express the whole or parts of the genes encoding the proteins or immunogenic fragments thereof according to the invention. Therefore, in a more preferred form of the embodiment relating to nucleic acid sequences, the invention relates to DNA fragments comprising a nucleic acid  
15 sequence according to the invention. A DNA fragment is a stretch of nucleotides that functions as a carrier for a nucleic acid sequence according to the invention. Such DNA fragments can e.g. be plasmids, into which a nucleic acid sequence according to the invention is cloned. Such DNA fragments are e.g. useful for enhancing the amount of DNA for use as a primer, for DNA-vaccination purposes  
20 and for expression of a nucleic acid sequence according to the invention, as described below.

An essential requirement for the expression of nucleic acid sequences is an adequate promoter functionally linked to the nucleic acid sequence, so that the  
25 nucleic acid sequence is under the control of the promoter. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression.

Therefore, an even more preferred form of this embodiment relates to a  
30 recombinant DNA molecule comprising a DNA fragment and/or a nucleic acid sequence according to the invention wherein the nucleic acid sequence according to the invention is placed under the control of a functionally linked promoter. This can be obtained by means of e.g. standard molecular biology techniques.

(Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

Functionally linked promoters are promoters that are capable of controlling the transcription of the nucleic acid sequences to which they are linked.

- 5 Such a promoter can be the native promoter of a novel gene according to the invention or another promoter of *Mycobacterium avium* subspecies *paratuberculosis*, provided that that promoter is functional in the cell used for expression. It can also be a heterologous promoter. When the host cells are bacteria, useful expression control sequences which may be used include the Trp
- 10 promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J., 1, 771-775, 1982); the bacteriophage lambda promoters and operators (Remaut, E. et al., Nucl. Acids Res., 11, 4677-4688, 1983); the  $\alpha$ -amylase (*B. subtilis*) promoter and operator,
- 15 termination sequences and other expression enhancement and control sequences compatible with the selected host cell.

- When the host cell is yeast, useful expression control sequences include, e.g.,  $\alpha$ -mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is
- 20 of vertebrate origin illustrative useful expression control sequences include the (human) cytomegalovirus immediate early promoter (Seed, B. et al., Nature 329, 840-842, 1987; Fynan, E.F. et al., PNAS 90, 11478-11482, 1993; Ulmer, J.B. et al., Science 259, 1745-1748, 1993), Rous sarcoma virus LTR (RSV, Gorman, C.M. et al., PNAS 79, 6777-6781, 1982; Fynan et al., supra; Ulmer et al., supra), the MPSV
- 25 LTR (Stacey et al., J. Virology 50, 725-732, 1984), SV40 immediate early promoter (Sprague J. et al., J. Virology 45, 773, 1983), the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983), the metallothionein promoter (Brinster, R.L. et al., Nature 296, 39-42, 1982), the heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985), the major late promoter of Ad2 and the  $\beta$ -actin
- 30 promoter (Tang et al., Nature 356, 152-154, 1992). The regulatory sequences may also include terminator and poly-adenylation sequences. Amongst the sequences that can be used are the well known bovine growth hormone poly-adenylation sequence, the SV40 poly-adenylation sequence, the human cytomegalovirus (hCMV) terminator and poly-adenylation sequences.

Bacterial, yeast, fungal, insect and vertebrate cell expression systems are very frequently used systems. Such systems are well-known in the art and generally available, e.g. commercially through Clontech Laboratories, Inc. 4030 Fabian Way, Palo Alto, California 94303-4607, USA. Next to these expression systems, parasite-based expression systems are attractive expression systems. Such systems are e.g. described in the French Patent Application with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W.: Public. Date 1 December 1993).

A still even more preferred form of this embodiment of the invention relates to Live Recombinant Carriers (LRCs) comprising a nucleic acid sequence encoding an *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment thereof according to the invention, a DNA fragment according to the invention or a recombinant DNA molecule according to the invention. These LRCs are micro-organisms or viruses in which additional genetic information, in this case a nucleic acid sequence encoding an *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment thereof according to the invention has been cloned. Cattle infected with such LRCs will produce an immunological response not only against the immunogens of the carrier, but also against the immunogenic parts of the protein(s) for which the genetic code is additionally cloned into the LRC, such as e.g. one or more of the novel *Mycobacterium avium* subspecies *paratuberculosis* proteins genes according to the invention.

As an example of bacterial LRCs, attenuated Salmonella strains known in the art can very attractively be used.

Also, live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998)).

Furthermore, LRC viruses may be used as a way of transporting the nucleic acid sequence into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali et al; Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E.P.A. 0473210A2), and Retroviruses (Valerio, D. et al; in Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik,

D.H. (Eds.), *Experimental Haematology today* - 1988. Springer Verlag, New York: pp. 92-99 (1989)).

The technique of *in vivo* homologous recombination, well-known in the art, can be used to introduce a recombinant nucleic acid sequence into the genome of a bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid sequence according to the invention in the host animal.

Finally another form of this embodiment of the invention relates to a host cell comprising a nucleic acid sequence encoding a protein according to the invention, a DNA fragment comprising such a nucleic acid sequence or a recombinant DNA molecule comprising such a nucleic acid sequence under the control of a functionally linked promoter. This form also relates to a host cell containing a live recombinant carrier comprising a nucleic acid molecule encoding an *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment thereof according to the invention.

A host cell may be a cell of bacterial origin, e.g. *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus* species, in combination with bacteria-based plasmids as pBR322, or bacterial expression vectors as the pEX-, pET-, pGEX-series, or with bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells like insect cells (Luckow et al; *Bio-technology* 6: 47-55 (1988)) in combination with vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. et al; *Cell* 32: 1033 (1983), mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

Another embodiment of the invention relates to the novel *Mycobacterium avium* subspecies *paratuberculosis* proteins and to immunogenic fragments thereof according to the invention.



The concept of immunogenic fragments will be defined below.

One form of this embodiment relates to a 28 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 2.

Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

The immunogenic fragments of the *Mycobacterium avium* subspecies *paratuberculosis* protein as depicted in SEQ ID NO: 2 and in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16 and 18 according to the invention as described below preferably have a length of at least 6, more preferably 7, 8, 9, 10, 12, 15, 20, 30 or even 40 amino acids, in that order of preference.

A still even more preferred form of this embodiment relates to this *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 1.

As was mentioned above, bacteria survive inside host cells, but extracellular episodes of infection in the lumen of the gastrointestinal tract do also occur. This implies that both cell-mediated and antibody-mediated immune responses play a role in adequate protection against disease. As is shown in the Examples, tests checking for both T-cell response and B-cell response have been used for the determination of the value of the proteins according to the invention as vaccine components.

Another form of this embodiment relates to a 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96%

homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 4.

5 Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ  
10 ID NO: 3.

Still another form of this embodiment relates to a 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at  
15 least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 6.

20 Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ  
25 ID NO: 5.

Again another form of this embodiment relates to a 47 kD *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at  
30 least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 8.

Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a 47 kD

- 5 *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 7.

- 10 One other form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 10.

- 15 Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

- 20 A still even more preferred form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 9.

- 25 Again an other form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 12.

- 30 Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 11.

Again another form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 14.

Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 13.

Again another form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96% homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 16.

Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

A still even more preferred form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 15.

Again another form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and to immunogenic fragments thereof, wherein the protein or immunogenic fragments have a sequence homology of at least 90%, preferably however 92%, more preferably 94 %, 95% or even 96%

homology, in that order or preference, to the amino acid sequence as depicted in SEQ ID NO: 18.

5 Even more preferred is a homology level of 97%, 98%, 99% or even 100% in that order of preference.

10 A still even more preferred form of this embodiment relates to a *Mycobacterium avium* subspecies *paratuberculosis* protein and immunogenic fragments of said protein, encoded by a nucleic acid sequence as depicted in SEQ ID NO: 17.

The level of protein homology can be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTP", that can be found at [www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

15 A reference for this program is Tatiana A. Tatusova, Thomas L. Madden FEMS Microbiol. Letters 174: 247-250 (1999). Matrix used: "blosum62". Parameters used are the default parameters:

Open gap: 11. Extension gap: 1. Gap x\_dropoff: 50.

20 It will be understood that, for the particular proteins embraced herein, natural variations can exist between individual *Mycobacterium avium* subspecies *paratuberculosis* strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid  
25 substitutions which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath et al in "The Proteins" Academic Press New York (1979). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and  
30 structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science, 227, 1435-1441, 1985) and determining the

functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain their immune reactivity.

- 5 This explains why *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention, when isolated from different field isolates, may have homology levels of about 70%, while still representing the same protein with the same immunological characteristics.

- Those variations in the amino acid sequence of a certain protein according to the  
10 invention that still provide a protein capable of inducing an immune response against infection with *Mycobacterium avium* subspecies *paratuberculosis* or at least against the clinical manifestations of the infection are considered as "not essentially influencing the immunogenicity".

- 15 When a protein is used for e.g. vaccination purposes or for raising antibodies, it is however not necessary to use the whole protein. It is also possible to use a fragment of that protein that is capable, as such or coupled to a carrier such as e.g. KLH, of inducing an immune response against that protein, a so-called immunogenic fragment. An "immunogenic fragment" is understood to be a  
20 fragment of the full-length protein that still has retained its capability to induce an immune response in a vertebrate host, e.g. comprises a B- or T-cell epitope. Shortly, an immunogenic fragment is a fragment that is capable of inducing an immunogenic response against an *Mycobacterium avium* subspecies *paratuberculosis* protein according to the invention. At this moment, a variety of  
25 techniques is available to easily identify DNA fragments encoding antigenic fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent NR. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-4002 (1984), J. Imm. Meth. 102, 259-274 (1987), the so-called PEPSCAN method is an easy to perform, quick and well-  
30 established method for the detection of epitopes; the immunologically important regions of the protein. The method is used world-wide and as such well-known to man skilled in the art. This (empirical) method is especially suitable for the detection of B-cell and T-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific protein

fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzofsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991). An immunogenic fragment usually has a minimal length of 6, more commonly 7-8 amino acids, preferably more than 8, such as 9, 10, 12, 15 or even 20 or more amino acids. The nucleic acid sequences encoding such a fragment therefore have a length of at least 18, more commonly 24 and preferably 27, 30, 36, 45 or even 60 nucleic acids.

Therefore, one form of still another embodiment of the invention relates to vaccines for combating *Mycobacterium avium* subspecies *paratuberculosis* infection, that comprise at least one *Mycobacterium avium* subspecies *paratuberculosis* protein or immunogenic fragments thereof, according to the invention as described above together with a pharmaceutically acceptable carrier.

Still another embodiment of the present invention relates to the *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention or immunogenic fragments thereof for use in a vaccine.

Again another embodiment of the present invention relates to the use of a nucleic acid sequence, a DNA fragment, a recombinant DNA molecule, a live recombinant carrier, a host cell or a protein or an immunogenic fragment thereof according to the invention for the manufacturing of a vaccine, more specifically a vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection.

One way of making a vaccine according to the invention is by growing the bacterium, followed by biochemical purification of an *Mycobacterium avium* subspecies *paratuberculosis* protein or immunogenic fragments thereof, from the bacterium or the supernatant. This is however a very time-consuming way of making the vaccine.

It is therefore much more convenient to use the expression products of a gene encoding an *Mycobacterium avium* subspecies *paratuberculosis* protein or immunogenic fragments thereof, according to the invention in vaccines. This is possible for the first time now because the nucleic acid sequences of genes encoding 9 novel *Mycobacterium avium* subspecies *paratuberculosis* proteins suitable as vaccine components is provided in the present invention.

Vaccines based upon the expression products of these genes can easily be made by admixing the protein according to the invention or immunogenic fragments thereof according to the invention with a pharmaceutically acceptable carrier as described below.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the protein according to the invention or immunogenic fragments thereof. Such vaccines, e.g. based upon a *Salmonella* carrier or a viral carrier e.g. a Herpesvirus vector have the advantage over subunit vaccines that they better mimic the natural way of infection of *Mycobacterium avium* subspecies *paratuberculosis*. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunization.

Vaccines can also be based upon host cells as described above, that comprise the protein or immunogenic fragments thereof according to the invention.

The vaccines according to the invention have an additional advantage over e.g. killed whole bacteria vaccines and live attenuated vaccines. Vaccines based upon the whole cell induce antibodies against all antigenic determinants, i.e. all epitopes present on the bacterium. Therefore, the antibody panel raised against such



vaccines is comparable to that raised after field infection. As a consequence, it is impossible to tell if an animal has been infected or has been vaccinated. The use of the proteins or immunogenic fragments thereof according to the invention, i.e. subunits of the whole bacterium, as vaccine components has the advantage that vaccinated animals only make antibodies against the administered subunits. A simple comparison of the antibody panel of suspected animals with that of vaccinated and field infected animals will immediately tell if the suspected animal was field-infected or vaccinated. For such tests, to be discussed below in more detail, a simple ELISA test is sufficient. Such vaccines based upon one or more subunits are known as marker vaccines: they are "marked" in the sense that they can be discriminated from field infection. Below, the concept of marker vaccines is discussed in more detail.

Very attractive marker vaccines are vaccines based upon the 9 kD protein or immunogenic fragments thereof, of which the sequence is depicted in SEQ ID NO: 6. The reason for this is the following: there is a relatively high level of cross-reactivity between antibodies raised against *Mycobacterium avium* subspecies *paratuberculosis* and *Mycobacterium bovis*. *Mycobacterium bovis* is i.a. the cause of bovine tuberculosis. The bacterium is also contagious for other animal species. Moreover, this disease is a zoonotic disease, i.e. it can be transferred to man. The World Health Organization (WHO) estimates that human tuberculosis (TB) incidence and deaths for 1990 to 1999 mounted to 88 million and 30 million, respectively, with most cases in developing countries. Zoonotic TB (caused by *Mycobacterium bovis*) is present in animals in most developing countries where surveillance and control activities are often inadequate or unavailable; therefore, many epidemiological and public health aspects of infection remain largely unknown.

The fact that *M. bovis* is contagious for other mammals is one of the reasons to attempt to eradicate *M. bovis*. One of the measures necessary to obtain this goal, is to eradicate cattle that is found positive for *M. bovis* in diagnostic tests such as the bovine PPD DTH test. And as mentioned above, due to cross-reactivity of sera against *M. bovis* and *Mycobacterium avium* subspecies *paratuberculosis* animals found positive in this test are eradicated, regardless the cause of infection.

Therefore, a diagnostic test that can clearly discriminate between vaccination with a subunit vaccine according to the invention and field infection with either *Mycobacterium bovis* or *Mycobacterium avium* subspecies *paratuberculosis* would be a very valuable tool.

5 It is one of the merits of the present invention that *Mycobacterium avium* subspecies *paratuberculosis* proteins were found that do not show cross reactivity with *Mycobacterium bovis* in a PPD test. The protein of which the amino acid sequence is given in SEQ ID NO: 6 belongs to these proteins.

10 Therefore, a preferred form of this embodiment relates to vaccines comprising a protein as depicted in SEQ ID NO: 6 or an immunogenic fragment thereof.

If a vaccine is wanted that does not necessarily have as a property that it can be used for marker purposes as described above, the addition of other proteins  
15 according to the invention or the 65 kD or 70 kD heat-shock protein, or immunogenic fragments thereof would be beneficial. Such combination vaccines enhance the efficacy of the vaccine, when compared to single vaccines.  
Therefore, in a more preferred form, a vaccine according to the invention comprises both the 9 kD protein one or more of the other proteins according to the invention  
20 or one of the heat shock proteins or immunogenic fragments thereof.

All vaccines described above contribute to active vaccination, i.e. they trigger the host's defence system.

25 Alternatively, antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the mammal to be vaccinated/protected. This method of vaccination, passive vaccination, is the vaccination of choice when a mammal is already infected, and there is no time to allow the natural immune response to be  
30 triggered. It is also the preferred method for vaccinating mammals that are prone to sudden high infection pressure and to immune compromised individuals. The administered antibodies against the protein according to the invention or immunogenic fragments thereof can in these cases interfere with *Mycobacterium*

*avium* subspecies *paratuberculosis*. This approach has the advantage that it decreases or stops *Mycobacterium avium* subspecies *paratuberculosis* development. Therefore, one other form of this embodiment of the invention relates to a vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection that  
5 comprises antibodies against an *Mycobacterium avium* subspecies *paratuberculosis* protein according to the invention or an immunogenic fragment of that protein, and a pharmaceutically acceptable carrier.

Still another embodiment of this invention relates to antibodies against an  
10 *Mycobacterium avium* subspecies *paratuberculosis* protein according to the invention or an immunogenic fragment of that protein.

Methods for large-scale production of antibodies according to the invention are also known in the art. Such methods rely on the cloning of (fragments of) the genetic  
15 information encoding the protein according to the invention in a filamentous phage for phage display. Such techniques are described i.a. at the "Antibody Engineering Page" under "filamentous phage display" at <http://aximt1.imt.uni-marburg.de/~rek/aepphage.html>, and in review papers by Cortese, R. et al., (1994) in Trends Biotechn. 12: 262-267., by Clackson, T. & Wells, J.A. (1994) in Trends  
20 Biotechn. 12: 173-183, by Marks, J.D. et al., (1992) in J. Biol. Chem. 267: 16007-16010, by Winter, G. et al., (1994) in Annu. Rev. Immunol. 12: 433-455, and by Little, M. et al., (1994) Biotechn. Adv. 12: 539-555. The phages are subsequently used to screen camelid expression libraries expressing camelid heavy chain  
25 antibodies. (Muyldermans, S. and Lauwereys, M., Journ. Molec. Recogn. 12: 131-140 (1999) and Ghahroudi, M.A. et al., FEBS Letters 414: 512-526 (1997)). Cells from the library that express the desired antibodies can be replicated and subsequently be used for large scale expression of antibodies.

Still another embodiment relates to a method for the preparation of a vaccine  
30 according to the invention that comprises the admixing of antibodies according to the invention and a pharmaceutically acceptable carrier.

An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has

been successful for many different proteins. (As reviewed in e.g. Donnelly et al., *The Immunologist* 2: 20-26 (1993)). More specifically, protection against *Mycobacterium avium* by DNA vaccination has been described by Velaz-Faircloth, M. et al., (*Infect. & Immun.* 67: 4243-4250 (1999)).

- 5 This way of vaccination is very attractive for the vaccination of cattle against *Mycobacterium avium* subspecies *paratuberculosis* infection. Therefore, still other forms of this embodiment of the invention relate to vaccines comprising nucleic acid sequences encoding a protein according to the invention or immunogenic fragments thereof, vaccines comprising DNA fragments that comprise such nucleic acid sequences or vaccines comprising recombinant DNA molecules according to
- 10 acid sequences or vaccines comprising recombinant DNA molecules according to the invention, and a pharmaceutically acceptable carrier. Preferably, nucleic acid sequences according to the invention encoding the 9 kD protein or an immunogenic fragment thereof and described in SEQ ID NO: 5 are used for vaccination, for reasons given above.
- 15 More preferably, such sequences are combined with sequences encoding another protein or an immunogenic fragment thereof according to the invention as described above.

- Examples of DNA plasmids that are suitable for use in a DNA vaccine according to
- 20 the invention are conventional cloning or expression plasmids for bacterial, eukaryotic and yeast host cells, many of said plasmids being commercially available. Well-known examples of such plasmids are pBR322 and pcDNA3 (Invitrogen). The DNA fragments or recombinant DNA molecules according to the invention should be able to induce protein expression of the nucleotide sequences.
- 25 The DNA fragments or recombinant DNA molecules may comprise one or more nucleotide sequences according to the invention. In addition, the DNA fragments or recombinant DNA molecules may comprise other nucleotide sequences such as immune-stimulating oligonucleotides having unmethylated CpG di-nucleotides, or nucleotide sequences that code for other antigenic proteins or adjuvating cytokines.
- 30 The nucleotide sequence according to the present invention or the DNA plasmid comprising a nucleotide sequence according to the present invention, preferably operably linked to a transcriptional regulatory sequence, to be used in the vaccine according to the invention can be naked or can be packaged in a delivery system.

Suitable delivery systems are lipid vesicles, iscoms, dendromers, niosomes, microparticles, especially chitosan-based microparticles, polysaccharide matrices and the like, (see further below) all well-known in the art. Also very suitable as delivery system are attenuated live bacteria such as *Salmonella* species, and  
5 attenuated live viruses such as Herpesvirus vectors, as mentioned above.

Still other forms of this embodiment relate to vaccines comprising recombinant DNA molecules according to the invention.

10 DNA vaccines can e.g. easily be administered through intradermal application such as by using a needle-less injector. This way of administration delivers the DNA directly into the cells of the animal to be vaccinated. Amounts of DNA in the range between 10 pg and 1000 µg provide good results. Especially if the DNA is self-replicating, minor amounts will suffice. Preferably, amounts in the microgram  
15 range between 1 and 100 µg are used.

In a further embodiment, the vaccine according to the present invention additionally comprises one or more antigens derived from cattle pathogenic organisms and viruses, antibodies against those antigens or genetic information  
20 encoding such antigens and/or a pharmaceutical component such as an antibiotic. Of course, such antigens, antibodies against such antigens, or genetic information can be of *Mycobacterium avium* subspecies *paratuberculosis* origin, such as e.g. another *Mycobacterium avium* subspecies *paratuberculosis* antigen. It can also be an antigen, antibodies or genetic information selected from another cow pathogenic  
25 organism or virus. Such organisms and viruses are preferably selected from the group of Bovine Herpesvirus, bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma* species, *Anaplasma* sp., *Neospora caninum*,  
30 *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma*, *E. coli*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Streptococcus dysgalactiae*.

As mentioned earlier, vaccines based upon one or more of the *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention are also very

suitable as marker vaccines. A marker vaccine is a vaccine that allows to discriminate between vaccinated and field-infected mammals e.g. on the basis of a characteristic antibody panel, different from the antibody panel induced by wild type infection. A different antibody panel is induced e.g. when an immunogenic protein present on a wild type *Mycobacterium avium* subspecies *paratuberculosis* is not present in a vaccine: the host will then not make antibodies against that protein after vaccination. Thus, a vaccine based upon any of the *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention would only induce antibodies against that specific protein, whereas a vaccine based upon a live wild-type, live attenuated or inactivated whole *Mycobacterium avium* subspecies *paratuberculosis* would induce antibodies against all or most of the bacterial proteins.

A simple ELISA test, having wells comprising any other *Mycobacterium avium* subspecies *paratuberculosis* protein except for a *Mycobacterium avium* subspecies *paratuberculosis* protein according to the present invention and wells comprising only one or more purified *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention suffices to test serum from cows and to tell if the cows are either vaccinated with the protein vaccine according to the invention or suffered from *Mycobacterium avium* subspecies *paratuberculosis* field infection.

All vaccines according to the present invention comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Methods for the preparation of a vaccine comprise the admixing of a protein or an immunogenic fragment thereof, according to the invention and/or antibodies against that protein or an immunogenic fragment thereof, and/or a nucleic acid sequence and/or a DNA fragment, a recombinant DNA molecule, a live recombinant carrier or host cell according to the invention, and a pharmaceutically acceptable carrier.

Vaccines according to the present invention may in a preferred presentation also contain an immunostimulatory substance, a so-called adjuvant. Adjuvants in

general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants frequently used in cow vaccines are muramyldipeptides,

lipopolysaccharides, several glucans and glycans and Carbopol<sup>(R)</sup> (a homopolymer).

5 The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the protein adheres, without being covalently bound to it. Such vehicles are i.a. bio-microcapsules, micro-alginates, liposomes and macrosols, all known in the art. Microparticles, more specifically those based upon chitosan, especially for use in oral vaccination are very suitable as vaccine vehicles.

10 A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span or Tween.

15 Antigens will preferably be combined with adjuvants that are readily available and that are registered for use in domestic animals and/or humans, e.g. aluminium hydroxide, a Th2-like modulating adjuvant.

Addition of CpG oligonucleotide sequences inside or outside the plasmid is also

20 preferred for improving protection.

Often, the vaccine is mixed with stabilisers, e.g. to protect degradation-prone proteins from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al; J.

25 Bacteriology 59: 509 (1950)), carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

In addition, the vaccine may be suspended in a physiologically acceptable diluent.

It goes without saying, that other ways of adjuvating, adding vehicle compounds or

30 diluents, emulsifying or stabilising a protein are also embodied in the present invention.

Vaccines according to the invention that are based upon the protein according to the invention or immunogenic fragments thereof can very suitably be administered

in amounts ranging between 1 and 100 micrograms of protein per animal, although smaller doses can in principle be used. A dose exceeding 100 micrograms will, although immunologically very suitable, be less attractive for commercial reasons.

- 5 Vaccines based upon live attenuated recombinant carriers, such as the LRC-viruses, parasites and bacteria described above can be administered in much lower doses, because they multiply themselves during the infection. Therefore, very suitable amounts would range between  $10^3$  and  $10^9$  CFU/PFU for both bacteria and viruses.

10

Vaccines according to the invention can be administered e.g. intradermally, subcutaneously, intramuscularly, intraperitoneally, intravenously, or at mucosal surfaces such as orally or intranasally.

15

For efficient protection against disease, a quick and correct diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection is important. Therefore it is another objective of this invention to provide diagnostic tools suitable for the detection of *Mycobacterium avium* subspecies *paratuberculosis* infection.

20

The nucleic acid sequences, the proteins and the antibodies according to the invention are also suitable for use in diagnostics.

- Therefore, another embodiment of the invention relates to nucleic acid sequences, proteins and antibodies according to the invention for use in diagnostics.

25

The nucleic acid sequences or fragments thereof according to the invention can be used to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* in cows. A sample taken from a mammal infected with *Mycobacterium avium*

30

subspecies *paratuberculosis* will comprise nucleic acid material derived from said bacterium, including nucleic acid sequences encoding for the protein according to the invention. These nucleic acid sequences will hybridize with a nucleic acid sequence according to the invention. Suitable methods for the detection of nucleic acid sequences that are reactive with the nucleic acid sequences of the present



invention include hybridization techniques including but not limited to PCR techniques and NASBA techniques. Thus the nucleic acid sequences according to the invention can be used to prepare probes and primers for use in PCR and or NASBA techniques.

- 5 A diagnostic test kit for the detection of *Mycobacterium avium* subspecies *paratuberculosis* may e.g. comprise tools to enable the reaction of *Mycobacterium avium* subspecies *paratuberculosis* nucleic acid isolated from the cows to be tested with these tools. Such tools are e.g. specific probes or (PCR-) primers, also referred to as primer fragments, based upon the nucleic acid sequences according to the
- 10 invention. If genetic material of *Mycobacterium avium* subspecies *paratuberculosis* is present in the animal, this will e.g. specifically bind to specific PCR-primers and, e.g. after cDNA synthesis, will subsequently become amplified in PCR-reaction. The PCR-reaction product can then easily be detected in DNA gel electrophoresis. Standard PCR-textbooks give methods for determining the length of the primers
- 15 for selective PCR-reactions with *Mycobacterium avium* subspecies *paratuberculosis* DNA. Primer fragments with a nucleotide sequence of at least 12 nucleotides are frequently used, but primers of more than 15, more preferably 18 nucleotides are somewhat more selective. Especially primers with a length of at least 20, preferably at least 30 nucleotides are very generally applicable. PCR-techniques
- 20 are extensively described in Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995).
- Nucleic acid sequences according to the invention or primers of those nucleic acid sequences having a length of at least 12, preferably 15, more preferably 18, even more preferably 20, 22, 25, 30, 35 or 40 nucleotides in that order of preference,
- 25 wherein the nucleic acid sequences or parts thereof have at least 70 % homology with the nucleic acid sequence as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 or 17 are therefore also part of the invention. Primers are understood to have a length of at least 12 nucleotides and a homology of at least 70%, more preferably 80%, 85%, 90%, 95%, 98%, 99% or even 100%, in that order of preference, with the
- 30 nucleic acid sequence as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 or 17. Such nucleic acid sequences can be used as primer fragments in PCR-reactions in order to enhance the amount of DNA that they encode or in hybridization reactions. This allows the quick amplification or detection on blots of specific nucleotide sequences

for use as a diagnostic tool for e.g. the detection of *Mycobacterium avium* subspecies *paratuberculosis* as indicated above.

Another test on genetic material is based upon *Mycobacterium avium* subspecies  
5 *paratuberculosis* material obtained from e.g. a swab, followed by classical DNA purification followed by classical hybridization with radioactively or colour-labelled primer fragments. Colour-labelled and radioactively labelled fragments are generally called detection means. Both PCR-reactions and hybridization reactions are well-known in the art and are i.a. described in Maniatis/Sambrook (Sambrook,  
10 J. *et al.* Molecular cloning: a laboratory manual. ISBN 0-87969-309-6).

Thus, one embodiment of the invention relates to a diagnostic test kit for the detection of *Mycobacterium avium* subspecies *paratuberculosis* nucleic acid sequences. Such a test comprises a nucleic acid sequence according to the invention  
15 or a primer fragment thereof.

A diagnostic test kit based upon the detection of antigenic material of the specific *Mycobacterium avium* subspecies *paratuberculosis* proteins according to the invention and therefore suitable for the detection of *Mycobacterium avium*  
20 subspecies *paratuberculosis* infection may i.a. comprise a standard ELISA test. In one example of such a test the walls of the wells of an ELISA plate are coated with antibodies directed against any of the proteins according to the invention. After incubation with the material to be tested, labelled anti- *Mycobacterium avium* subspecies *paratuberculosis* antibodies are added to the wells. A colour reaction  
25 then reveals the presence of antigenic material from *Mycobacterium avium* subspecies *paratuberculosis*.

Therefore, still another embodiment of the present invention relates to diagnostic test kits for the detection of antigenic material of *Mycobacterium avium* subspecies *paratuberculosis*. Such test kits comprise antibodies against a protein according to  
30 the invention or a fragment thereof according to the invention.

A diagnostic test kit based upon the detection in serum of antibodies against a protein of *Mycobacterium avium* subspecies *paratuberculosis* according to the invention and therefore suitable for the detection of *Mycobacterium avium*

subspecies *paratuberculosis* infection may i.a. comprise a standard ELISA test. In such a test the walls of the wells of an ELISA plate can e.g. be coated with an *Mycobacterium avium* subspecies *paratuberculosis* protein according to the invention. After incubation with the material to be tested, labelled anti-bodies  
5 against that protein are added to the wells. A lack of colour reaction then reveals the presence of antibodies against *Mycobacterium avium* subspecies *paratuberculosis*.

Therefore, still another embodiment of the present invention relates to diagnostic test kits for the detection of antibodies against *Mycobacterium avium* subspecies  
10 *paratuberculosis*. Such test kits comprise an *Mycobacterium avium* subspecies *paratuberculosis* protein according to the invention or a fragment thereof according to the invention.

The design of the immunoassay may vary. For example, the immunoassay may be  
15 based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labelled antibodies; the labels may be, for example, enzymes, fluorescent-, chemoluminescent-, radio-active- or dye molecules.

Suitable methods for the detection of antibodies reactive with a protein according  
20 to the present invention in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescence test (IFT) and Western blot analysis.

The proteins or immunogenic fragments thereof according to the invention e.g. expressed as indicated above can be used to produce antibodies, which may be  
25 polyclonal, monospecific or monoclonal (or derivatives thereof). If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are well-known in the art (e.g. Mayer and Walter, eds. *Immunochemical Methods in Cell and Molecular Biology*, Academic Press, London, 1987).

Monoclonal antibodies, reactive against the protein according to the invention or  
30 an immunogenic fragment thereof according to the present invention, can be prepared by immunizing inbred mice by techniques also known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975).

It was found, that *Mycobacterium avium* subspecies *avium* has an extremely high homology with *Mycobacterium avium* subspecies *paratuberculosis*. *Mycobacterium avium* subspecies *avium* is found, with a significantly increasing incidence, in pigs but also more and more frequently in humans, especially in immune deficient

5 humans such as HIV-positives.

The proteins according to the invention as mentioned above can therefore equally well be used for vaccination purposes against *Mycobacterium avium* subspecies *avium* in both pigs and humans, but also for diagnostic purposes in pigs and humans.

**EXAMPLES.**Example 1

*Screening of expression library.* In order to identify and characterize antigens in *M. avium subsp. paratuberculosis* for use in diagnostics, therapeutics and vaccines, a genomic expression library was constructed using the lambda TripleEx expression vector according to the Clontech manual (pT3003-1) and Stratagene Gigapac III Gold Packaging manual. Briefly, bacterial genomic DNA isolated from *M. avium subsp. paratuberculosis* strain 316F was partially digested with *Tsp509I* and fragments of average size of 2.5 kilobase pairs, obtained by sucrose gradient centrifugation, were ligated to EcoRI-digested, dephosphorylated lambda TripleEx arms. The packaging reaction was carried out using Gigapack III Gold Packaging Extract and host strain *E. coli* XL1Blue (Clontech (S0924)). After plating of the library, immunoscreening of approximately 10<sup>6</sup> phage plaque's was carried out with 1) a positive bovine serum (designated as 3869) and 2) specific anti-*Mycobacterium avium* subspecies *paratuberculosis* monoclonal antibodies. This resulted in the selection of 125 positive lambda TripleEx recombinants. Hundred and seventeen of these 125 positive phage recombinants were successfully converted to plasmid (pTripleEx) recombinants using the protocol described in the Clontech manual (PT3003-1).

DNA sequencing of these 117 pTripleEx recombinants allowed them to be categorized into different antigen groups with each group expressing a different antigenic protein or fragment thereof. SEQ 2, 4 and 6 were found in recombinants isolated with serum 3869, SEQ 8 in recombinants isolated with monoclonal antibodies to FabG4, and SEQ 10,12,14,16 and 18 in recombinants isolated with 5 respective monoclonal antibodies (13.67.1A; 10.65.3B; 13.67.2A; 10.32.3B; and 10.66.4B) directed to 5 antigenic molecules of *M. avium subsp. paratuberculosis*. Blast searches against various data bases containing mycobacterial genomic information allowed further characterization of a number of the antigenic polypeptides and their encoding genes. Except for hsp65 and hsp70 heat shock protein antigens found and described in SEQ. ID. No: 19 and 21, none of the here provided antigenic fragments have so far been identified as a for *M. avium subsp. paratuberculosis* relevant antigen or figure among the already known antigens discussed above for *M. avium subsp. paratuberculosis*.

### Example 2

*Proteomics approach for identification of relevant proteins.*

a) Sample preparation. Cells were harvested from a culture of *M. avium* sups.

5 *paratuberculosis* 316F in Watson and Reed culture medium by centrifugation. The cell pellet was washed once with PBS (10 g pellet /40ml PBS), and stored at  $-80^{\circ}\text{C}$  in 5 ml portions. After thawing, each sample was washed twice with 100 ml cold PBS ( $4^{\circ}\text{C}$ ), and suspended in 5 ml cold PBS. Proteinase inhibitors were added (pepstatin 12,5 ug; leupeptin 25 ug, Pefabloc<sup>TM</sup>SC 125 ug; aprotinine 5 ug); and the  
10 suspension was sonicated using a Branson sonifier 250 for 10 ' at 100 % output with 50 % interval, on ice. Subsequently Ureum (9M), DTT (70 mM), and Triton X-100 (2%) was added, and the solution was kept at RT for 30' with occasional shaking. The suspension was subsequently centrifuged for 15' at 5,000 g at  $16^{\circ}\text{C}$ , and again for 30' at 100,000 g at  $16^{\circ}\text{C}$ . The resulting samples were subsequently  
15 treated with the PlusOne 2-D clean up kit (Amersham Biosciences) to remove trace amount of salts, polysaccharides, nucleic acids, and lipids according to the protocol provided by the manufacturer. The protein concentration of the sample was determined using RC DC Protein Assay (Bio-Rad Laboratories), and the samples were stored at  $-80^{\circ}\text{C}$  until 2D-PAGE. Typically, approximately 100 ug of protein  
20 sample was used in 2D-PAGE when followed by silver staining or immune blotting, and up to 1500 ug of protein sample was used when 2D-PAGE was followed by Coomassie Brilliant Blue staining.

b) 2D-PAGE. Iso-electric focusing (IEF) was carried out using the Ettan IPGphor  
25 Iso-electric Focusing system (Amersham Biosciences) with rehydration of IPGphor strips and IEF using ceramic strip holders according to the protocols provided by the manufacturer. Typically, rehydration and protein loading of 24 cm strips was carried out by adding 450 ul of the protein sample in rehydratation buffer containing 1.4 mg DTT and 0.5 % ul IPG-buffer followed by incubation O/N at  $20^{\circ}\text{C}$ .  
30 Subsequently, IEF was carried out according to instructions provided by the manufacturers. After IEF, strips can be stored at  $-20^{\circ}\text{C}$  until second-dimension PAGE. For second dimension PAGE, strips were equilibrated by shaking in 14 ml equilibration buffer containing 140 mg DTT for 15 ' at RT, and subsequently by shaking in 15 ml equilibration buffer containing 350 mg Iodoacetamide for 15 ' at

RT. Subsequently, strips were briefly dipped in cathode buffer and applied for second dimension PAGE. Electrophoresis was carried out in 12.5 % Ettan Dalt II gels (26 x20 cm; 1 mm thickness) using a Ettan Dalttwelve large format vertical system according to the instructions provided by the manufacturers (Amersham Biosciences). For silver staining, gels were fixed in a 40 % ethanol, 10 % acetic acid solution and stained using plus one silver stain kit (Amersham Biosciences). For Coomassie Brilliant Blue staining, gels were stained using PhastGel Blue R (Amersham Biosciences) according to the instructions provided by the manufacturers. For immune blotting, proteins were transferred to nitrocellulose (Nitrocellulose BA85; Schleicher and Schuell) using the Trans-Blot SD Semi Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories) according to the instructions provided by the manufacturers. Recognition of protein spots by monoclonal or polyclonal antibodies was carried out using a standard protocol using PBS buffer with 5 % skimmed milk, rabbit anti-mouse or anti-cow antibodies conjugated to horse radish peroxidase, and peroxide, TMB and DONS as substrate.

Results: as follows from figure 1, this approach led to the detection of two immunologically highly relevant proteins.

The one protein is a 60 kD *Mycobacterium avium* subspecies *paratuberculosis* protein having a pI between 5.60 and 6.15. This protein is visible as a horizontal row of about 5 spots in figure 1 b and d.

The other protein is a 33 kD *Mycobacterium avium* subspecies *paratuberculosis* protein having a pI between 4.20 and 4.75. This protein is visible as a horizontal row of about 3 spots in figure 1 a and d.

### Example 3

*Recognition of the 14 kD protein, the 9 kD protein and Hsp70 and Hsp65 by T-cells from vaccinated and infected goats.*

Stimulation of T-cells in peripheral blood samples from goats experimentally infected with *M. avium* subsp. *paratuberculosis* was detected using a bovine interferon gamma test (BOVIGAM; CSL laboratories Parkville, Victoria, Australia) according to protocol provided by the manufacturers. The following antigens were added to 1.5 ml of whole blood: recombinant purified 14 kD protein, 9 kD protein, Hsp70 and Hsp65 (0.5 and 5 ug), and three PPDs (3 ug) derived from *M. bovis* strain AN5 (produced by ID-Lelystad, The Netherlands), derived from *M. avium*

5 *subsp. avium* strain D4 (produced by ID-Lelystad, The Netherlands), and derived from *M. avium subsp. paratuberculosis* strains 3+5/C (produced by ID-Lelystad, The Netherlands), respectively. Each of these antigens were tested at three consecutive times using bi-weekly samples from 9 goats that approximately two years earlier were 12 times orally infected with 1 ml (OD<sub>660</sub>=0.059) of *M. avium subsp. paratuberculosis* strain DSU no. 405650 (over a 4 week period: each week on Mondays, Wednesdays and Fridays). Five of these animals (188-193) were vaccinated with 0.5 ml of an experimental killed vaccine based on attenuated *M. avium subsp. paratuberculosis* strain 316F (produced by ID-Lelystad, The Netherlands) 4 weeks before infection. Absorbance values  $\geq 0.1$  (when corrected for background values) and  $\geq 2$  times background values were regarded to show increased production of interferon-gamma due to the presence of antigen.

15 Results: all recombinant antigens tested induced increased interferon-gamma production in at least one of the animals. This shows that they all play a role in T-cell-mediated immune response. A typical experiment is shown in Table 1. Five out of 9 animals showed an increased response to the 9 kD antigen (56 %), three out of nine animals (33%) to the 14 kD, 8 out of 9 (90%) to hsp65, and three out of nine (33%) to hsp70.



Table 1

Exp 5. 040901	188	189	190	191	193	194	195	196	198
AG									
Bovis PPD (3 ug)	1.778	1.089	0.426	1.167	0.475	0.174	0.162	0.054	0.701
Avium PPD (3 ug)	3.484	1.795	1.348	3.475	3.114	0.731	0.655	0.099	2.466
Paratb PPD (3 ug)	3.501	2.664	2.187	> 4	3.319	1.919	1.339	0.254	3.078
14 kD 0.5 ug	0.022	-0.017	0.102	-0.023	0.022	0.103	0.004	-0.008	0.392
14 kD 5 ug	0.018	0.023	0.175	-0.017	0.011	0.151	0.131	-0.002	0.333
9 kD 0.5 ug	0.103	0.424	0.213	0.006	0.149	0.409	0.025	0.036	0.022
9 kD 5 ug	0.139	1.169	0.262	0.009	0.065	1.712	0.243	0.045	0.107
47 kD 0.5 ug	-0.022	-0.020	-0.012	-0.040	-0.002	-0.007	-0.006	-0.007	-0.002
47 kD 5 ug	-0.006	0.001	-0.007	-0.041	-0.010	0.019	0.009	-0.005	-0.010
Hsp70 0.5 ug	0.092	-0.012	0.143	-0.006	-0.011	0.286	0.007	-0.010	0.070
Hsp70 5 ug	0.174	0.032	0.220	0.023	0.001	0.969	0.094	0.001	0.187
Hsp65 0.5 ug	0.633	0.945	0.390	0.105	0.111	0.247	0.456	-0.001	0.121
Hsp65 5 ug	1.069	0.949	0.925	0.188	0.357	1.086	0.599	0.049	0.213

Example 4.

*Immunisation of calves with the 14 kD, 9 kD, 47 kD, 70 kD and 65 kD proteins, detection of specific antibody responses, and detection DTH-reactivity.*

## a) Immunisation.

- 5 In order to evaluate the immunogenicity of the 14 kD, 9 kD, 47 kD, 70 kD and 65 kD proteins and the ability to induce a (cross-reactive) DTH response to PPD derived from *M. bovis*, calves were immunised as follows: 1) with killed whole cell vaccine based on attenuated *M. avium* subspecies *paratuberculosis* 316F (produced by ID-Lelystad, The Netherlands; 4 animals) 2) purified recombinant 14 kD protein
- 10 in a W/O adjuvant (4 animals) 3) purified recombinant 9 kD protein in a W/O adjuvant (4 animals) 4) purified recombinant 47 kD protein in a W/O adjuvant (4 animals) 5) purified recombinant Hsp70 in a W/O adjuvant (2 animals) 6) purified recombinant Hsp65 in a W/O adjuvant (2 animals) 7) a W/O adjuvant alone (3 animals). Prime and boost immunisations of the following amounts of antigen were
- 15 given at day 0 and day 127:

Antigen	prime (ug)	booster (ug)
14 kD	207	259
9 kD	156	236
47 kD	273	305
70 kD	348	342
65 kD	681	491

- Immunisations with the experimental vaccine were given at day 0 (1 ml) and 127 (0.5 ml). Serum samples were taken at day 52 and day 178. DTH tests were done at
- 20 day -56 (to establish DTH status of animals before immunisation), and at day 52 and 178.

## b) Antibody detection in serum.

- Immunogenicity of the antigens and their presence in PPD was established by
- 25 detection of total IgG antibodies in serum samples from one representative animal from each immunisation group using a standard SDS-PAGE and immunoblotting protocol where lanes were loaded with 2.5 ug of purified recombinant 14 kD, 9 kD, 47 kD, 65 kD and 70 kD proteins, and 2.5 ug of various extracts (whole cell sonicate

and PPD derived from *M. bovis* strain AN5; whole cell sonicate from *M. avium paratuberculosis* strain B854; PPD derived from *M. avium paratuberculosis* strains 3+5/C).

5 Results: Antibodies in all representative sera detected the corresponding recombinant 14, 9, 47, 65, and 70 kD proteins (Figure 2 panels A-E, lanes 1); Antibodies to recombinant 14 kD protein failed to recognise a corresponding antigen in *M. bovis* and *M. avium paratuberculosis* whole cell sonicates and PPDs (Figure 2 panel A, lanes 2-4); Antibodies to recombinant 9 kD protein recognised a  
10 corresponding antigen in *M. bovis* and *M. avium paratuberculosis* whole cell sonicates and *M. avium paratuberculosis* PPD, but did not recognise a corresponding protein in *M. bovis* PPD (Figure 2 panel B, lanes 2-4); Antibodies to recombinant 47 kD protein recognised a (weak) corresponding antigen in *M. bovis* and *M. avium paratuberculosis* whole cell sonicates, but failed to recognise a  
15 corresponding band in PPDs (Figure 2 panel C, lanes 2-4); Antibodies to recombinant 65 and 70 kD proteins recognised a corresponding antigen in *M. bovis* and *M. avium paratuberculosis* whole cell sonicates and PPDs (Figure 2 panels D and E, lanes 2-4).

20

Additionally, immunogenicity of the antigens was established by detection of total IgG antibodies in serum samples using a standard ELISA protocol where wells were coated with 5 ug of various extracts (whole cell sonicates, KCL extracts, secreted proteins) of various *M. avium paratuberculosis* strains (B854, 5255, 316F, 3+5/C, Teps) or *M. bovis* strain AN5, and with 5 ug of recombinant purified 14 kD, 9 kD, 47 kD, 70 kD and 65 kD proteins. Arbitrarily, titers above 1/80 at OD=1.0  
25 were regarded as indicative for a positive response.

Results: strong specific antibody responses were detected to the 14 kD, 47 kD, 70 kD and 65 kD proteins after primary and boost immunisation (titers > 640; Table 2 and 3). Specific antibody titers to the 9 kD protein were detected after a boost immunisation (titers 160; Table 2 and 3).

30

Table 2.

		Serum titers at OD= 1.0 (day 52)										
		B85 4 For m Cell s	525 5 US O	3+5/ C Joh n.	316 F Exc r.	Tep s KC L	AN5 KC L	14 kD	9 kD	47 kD	Hsp 70	Hsp 65
Paratb	840	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	<5	5	40	<5
	643 1	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	<5	<5	40	10
	702 9	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	<5	<5	20	5
	929 3	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	5	<5	40	10
14 kD	595 5	10	20	<5	10	20	40	320	<5	<5	5	20
	700 2	<5	5	<5	<5	10	20	>64 0	<5	40	<5	10
	854 6	40	40	10	20	160	20	>64 0	<5	<5	5	10
	950 6	5	5	<5	10	40	40	>64 0	<5	<5	<5	20
9 kD	172 8	<5	5	<5	5	10	<5	<5	<5	<5	<5	10
	743 0	5	<5	<5	<5	40	10	<5	<5	5	10	10
	811 6	<5	80	5	5	10	5	<5	<5	<5	<5	<5
	878 3	20	80	20	20	40	40	<5	<5	5	5	10

[illegible]

Table 3.

		Serum titers at OD= 1.0 (day 178)										
		B85 4 For m Cell s	525 5 US O	3+5/ C Joh n.	316 F Exc r.	C KC L	AN5 KC L	14 kD	9 kD	47k D	Hsp 70	Hsp 65
Paratb	840	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	5	5	10	320	20
	643 1	>64 0	>64 0	>64 0	>64 0	<64 0	>64 0	<5	10	5	40	20
	702 9	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	5	5	10	5
	929 3	>64 0	>64 0	>64 0	>64 0	>64 0	>64 0	<5	10	10	20	40
14 kD	595 5	20	80	<5	20	320	5	>64 0	5	20	10	40
	700 2	<5	20	<5	<5	320	<5	>64 0	10	10	<5	20
	854 6	10	80	5	20	80	10	>64 0	40	5	5	20
	950 6	5	40	<5	10	160	5	>64 0	20	10	10	20
9 kD	172 8	<5	10	<5	20	80	<5	10	<5	40	10	80
	743 0	<5	40	<5	5	80	20	5	160	20	<5	40
	811 6	<5	20	<5	<5	160	40	40	160	80	10	80
	878	40	80	20	80	320	10	10	160	10	5	40

[illegible]

## c) DTH reactivity.

Delayed type hypersensitivity (DTH) reactivity was done according to EU-directive 64/432 (as amended by directive 97/12 and 98/46). Briefly, 2000 IE avian PPD, and 2000 and 5000 IE bovine PPD were injected and the increase in skin thickness after 72 hours detected. An increase of more than 2 mm is regarded as a positive DTH response. In the whole cell killed paratuberculosis vaccine group, bovine PPD reactivity was detected in all four animals both after prime and boost immunisation (Table 4). Bovine PPD reactivity was detected in animals vaccinated with the 14 kD protein (1/4) and Hsp65 (1/2) after prime immunisation, and the 47 kD protein (1/4) after boost immunisation (Table 4). Bovine PPD reactivity was not detected in the groups immunised with the 9 kD protein and the 70 kD protein (Table 4).

Results: the 9 kD and Hsp70 proteins do not give a response in a DTH PPD test. In case a vaccine is needed that does contribute to protection against infection and additionally does not cross-react with PPD in a DTH PPD test, the novel 9 kD protein according to the present invention would be the vaccine component of choice, preferably in combination with Hsp70.





47 kD	296 8	0	0		0	0	0		0	0	0
	296 9	0	0		0	0	0		1	0	0
	384 7	0	0		0	0	0		0	0	0
	391 1	0	0		0	0	0		1	0	4
Hs p70	702 0	0	0		1.5	0	0		0	0	0
	704 9	0	0		0	0	0		0	0	0
Hs p65	310 5	0	0		<b>10</b>	0.5	<b>6</b>		2	0	0
	950 5	0	0		0	0	0		0	0	0
Adj u	172 7	0	0		0	0	0		0	0	0
	384 8	0	0		2.0	0.5	0		1	0	0
	742 9	0	0		0	0	0		0	0	0
No ne	835 4	<b>2.7</b>	0		<b>2.5</b>	0	0		NT	NT	NT
	873 8	0	0		0	0	0		0	0	0

ADTH > 2 mm is indicated in bold.

NT, Not tested; animal removed during experiment.

### Legend to the figures

Figure 1.A shows in a Western blot of a 2D-gel the presence of a 33 kD protein  
5 having a pI between 4.20 and 4.75 and visible as a horizontal row of about 3 spots.

Figure 1.B shows in a Western blot of a 2D-gel the presence of a 60 kD protein  
having a pI between 5.60 and 6.15 and visible as a horizontal row of about 5 spots.

10 Figure 1.C shows the 2D-gel stained with Coomassie Brilliant Blue, in which the  
specific spots of both the 33 kD and the 60 kD protein are visible.

Figure 1.D shows the 2D-gel stained with silver staining, in which the specific  
spots of both the 33 kD and the 60 kD protein are again visible.

15

### Figure 2.

Immunoblots with serum samples (day 178) from animals immunised with  
recombinant purified 14 kD protein (panel A), with recombinant purified 9 kD  
protein (Panel B), with recombinant 47 kD (panel C), with recombinant purified 70  
20 kD (panel D), and with recombinant 65 kD (panel E).

Lane 1, Recombinant purified protein of 14 (A), 9 (B), 47 (C), 70 (D) or 65 kD (E).

Lane 2, *M. bovis* strain AN5 whole cell sonicate.

Lane 3, *M. avium paratuberculosis* strain B854 whole cell sonicate.

Lane 4, *M. avium paratuberculosis* strains 3+5/C derived PPD.

25 Lane 5, *M. bovis* strain AN5 derived PPD.

30

35

## Claims

- 1) Nucleic acid sequence encoding a 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
5 an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 5
- 2) Nucleic acid sequence encoding a 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
10 an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 3
- 3) Nucleic acid sequence encoding a 28 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
15 an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 1
- 4) Nucleic acid sequence encoding a 47 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
20 an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 7
- 5) Nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
25 an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 9
- 6) Nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes  
30

an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 11

- 5 7) Nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 13
- 10 8) Nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 15
- 15 9) Nucleic acid sequence encoding a *Mycobacterium avium* subspecies *paratuberculosis* protein or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein, said nucleic acid sequence or said part thereof having at least 85%, preferably 90 %, more preferably 95 % homology with the nucleic acid sequence of the *Mycobacterium avium* subspecies *paratuberculosis* protein gene as depicted in SEQ ID NO: 17
- 20 10) Nucleic acid sequence encoding a 60 kD *Mycobacterium avium* subspecies *paratuberculosis* protein having a pI of 5.60-6.15 or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein.
- 25 11) Nucleic acid sequence encoding a 33 kD *Mycobacterium avium* subspecies *paratuberculosis* protein having a pI of 4.20-4.75 or a part of said nucleic acid sequence that encodes an immunogenic fragment of said protein.
- 12) DNA fragment comprising a nucleic acid sequence according to claim 1-11.
- 30 13) Recombinant DNA molecule comprising a nucleic acid sequence according to claim 1-11 or a DNA fragment according to claim 12, under the control of a functionally linked promoter.

- 14) Live recombinant carrier comprising a nucleic acid sequence according to claim 1-11, a DNA fragment according to claim 12 or a recombinant DNA molecule according to claim 13.
- 5 15) Host cell comprising a nucleic acid sequence according to claim 1-11, a DNA fragment according to claim 12, a recombinant DNA molecule according to claim 13 or a live recombinant carrier according to claim 14.
- 10 16) A 9 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 %to the amino acid sequence as depicted in SEQ ID NO: 6.
- 15 17) A 14 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 %to the amino acid sequence as depicted in SEQ ID NO: 4.
- 20 18) A 28 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 %to the amino acid sequence as depicted in SEQ ID NO: 2.
- 25 19) A 47 kD *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 %to the amino acid sequence as depicted in SEQ ID NO: 8.
- 30 20) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 %to the amino acid sequence as depicted in SEQ ID NO: 10.
- 21) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%,

preferably 92 %, more preferably 94 % to the amino acid sequence as depicted in SEQ ID NO: 12.

22) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 % to the amino acid sequence as depicted in SEQ ID NO: 14.

23) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 % to the amino acid sequence as depicted in SEQ ID NO: 16.

24) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, characterized in that said protein or immunogenic fragment thereof has a sequence homology of at least 90%, preferably 92 %, more preferably 94 % to the amino acid sequence as depicted in SEQ ID NO: 18.

25) A *Mycobacterium avium* subspecies *paratuberculosis* 60 kD protein having a pI of 5.60-6.15 or an immunogenic fragment of said protein.

26) A *Mycobacterium avium* subspecies *paratuberculosis* 33 kD protein having a pI of 4.20-4.75 or an immunogenic fragment of said protein.

27) A *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment of said protein, according to claims 16-26, characterized in that said protein or immunogenic fragment is encoded by a nucleic acid sequence according to claim 1-11.

28) An *Mycobacterium avium* subspecies *paratuberculosis* protein or an immunogenic fragment thereof, according to claims 16-26, or a nucleic acid sequence according to claims 1-11, for use in a vaccine.

29) Use of a nucleic acid sequence according to claim 1-11, a DNA fragment according to claim 12, a recombinant DNA molecule according to claim 13, a live recombinant carrier according to claim 14, a host cell according to claim 15 or a protein according to claims 16-26 or an immunogenic fragment thereof for the manufacturing of a vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection.

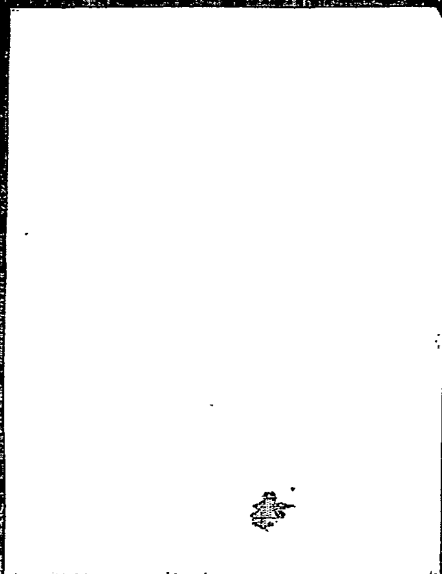
- 30) Vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection, characterized in that said vaccine comprises at least one *Mycobacterium avium* subspecies *paratuberculosis* protein according to claims 16-26 or an immunogenic fragment of said protein and a pharmaceutically acceptable carrier.
- 31) Vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection, characterized in that said vaccine comprises a nucleic acid sequence according to claims 1-11, a DNA fragment according to claim 12, a recombinant DNA molecule according to claim 13, a live recombinant carrier according to claim 14 or a host cell according to claim 15 and a pharmaceutically acceptable carrier.
- 32) Vaccine for combating *Mycobacterium avium* subspecies *paratuberculosis* infection, characterized in that said vaccine comprises antibodies against a protein according to claims 16-26 or an immunogenic fragment of said protein and a pharmaceutically acceptable carrier.
- 33) Vaccine according to claims 30-32, characterized in that said vaccine comprises an adjuvant.
- 34) Vaccine according to claims 30-33, characterized in that said vaccine comprises an additional antigen derived from a virus or micro-organism pathogenic to cattle, an antibody against said antigen or genetic information encoding said antigen.
- 35) Vaccine according to claim 34, characterized in that said virus or micro-organism pathogenic to cattle is selected from the group of Bovine Herpesvirus, bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma* species, *Anaplasma* sp., *Neospora caninum*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma*, *E. coli*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Streptococcus dysgalactiae*.
- 36) Method for the preparation of a vaccine according to claims 30-35, said method comprising the admixing of a nucleic acid sequence according to claims 1-11, a DNA fragment according to claim 12, a recombinant DNA molecule according to claim 13, a live recombinant carrier according to claim 14, a host cell according to claim 15, a protein according to claims 16-26 or



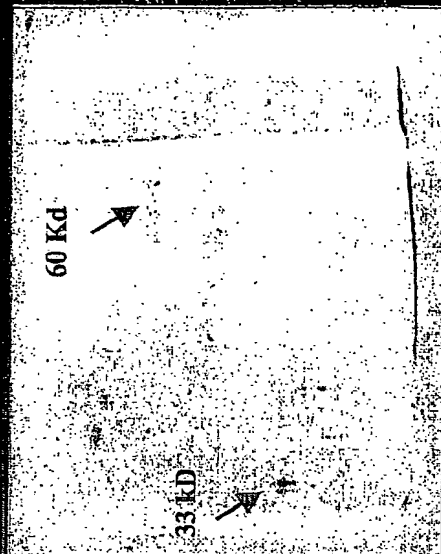
antibodies against a protein according to claims 16-26, and a pharmaceutically acceptable carrier.

- 5 37) A diagnostic kit comprising suitable detection means and a nucleic acid sequence according to claims 1-11 or a primer thereof, a protein according to claims 16-26 or an immunogenic fragment thereof, or antibodies that are reactive with a protein according to claims 16-26.

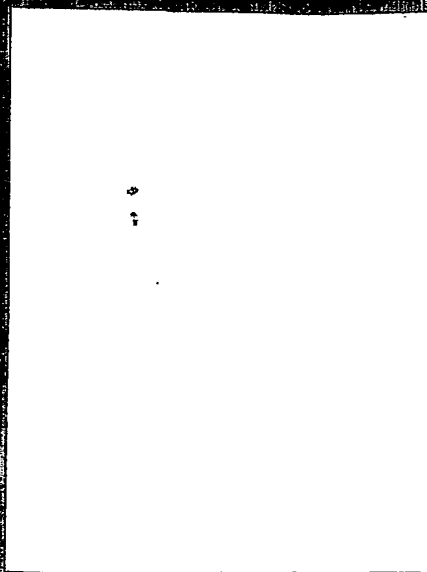
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C. Coomassie Brilliant Blue



B. MoAb 13.67.2A 1:50 (60 KD)



D. Silver

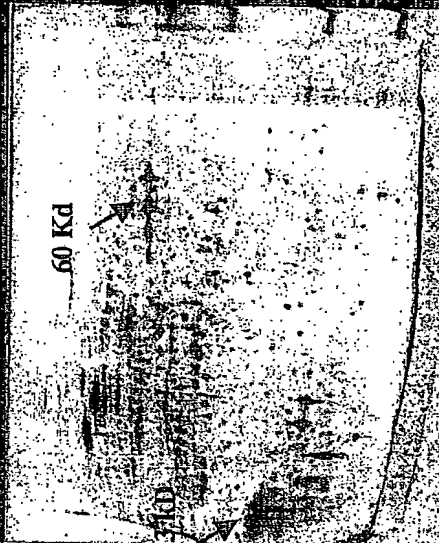
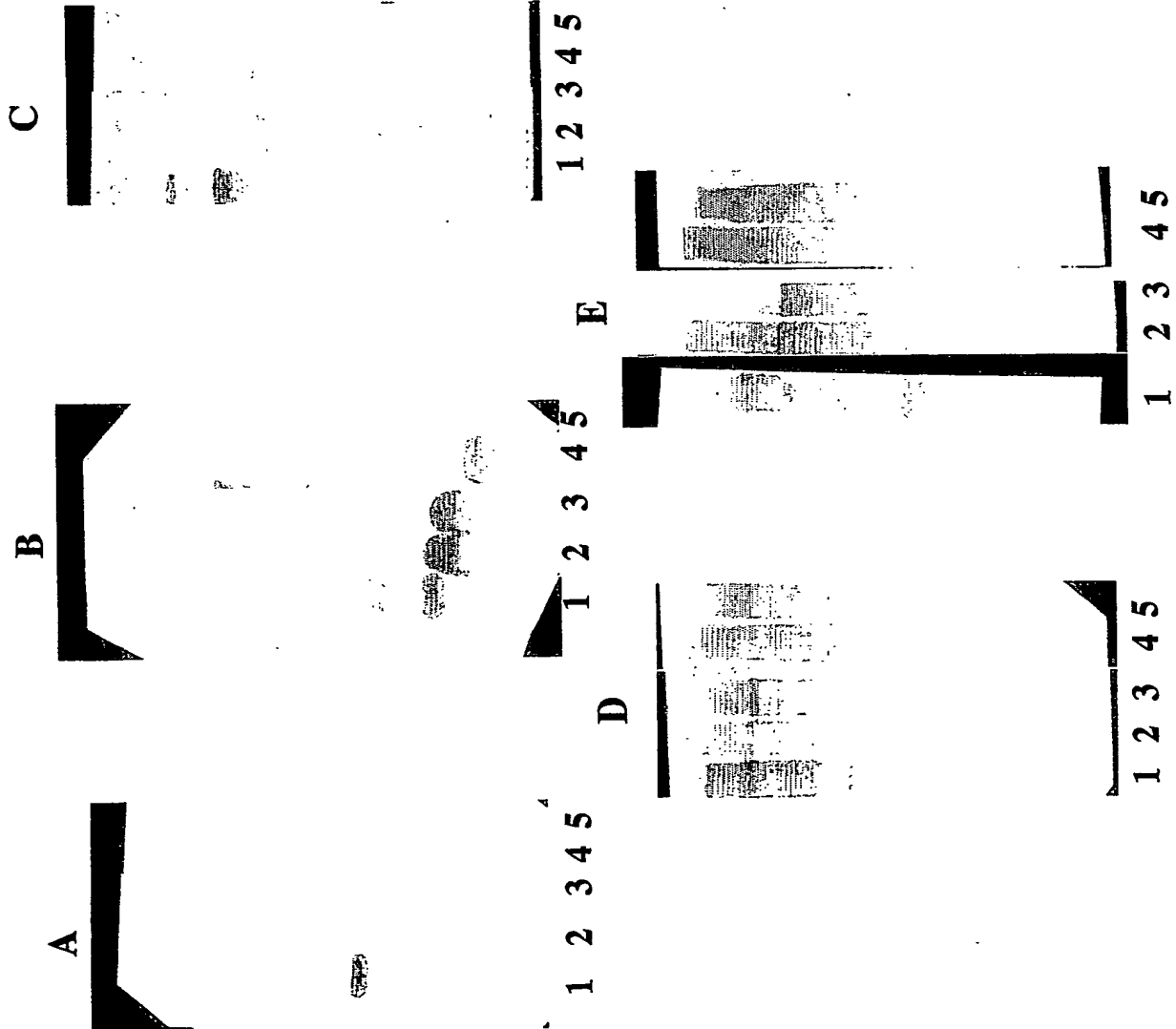


Figure 1



## SEQUENCE LISTING

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6/29

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7/29

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Tyr Arg Pro Gly Asp Pro Pro Leu Ala Gly Ser Leu Leu Ile Gly Gly	
40 45 50	
gag ggc cgc gtg gtc gag ccg ctg ccg gcg gcg ctg gcc aag gac tac	249
Glu Gly Arg Val Val Glu Pro Leu Arg Ala Ala Leu Ala Lys Asp Tyr	
55 60 65	
gac ctg gtc ggc aac aac ctg ggc ggg cgc tgg gcc gac cgg ttc ggc	297
Asp Leu Val Gly Asn Asn Leu Gly Gly Arg Trp Ala Asp Arg Phe Gly	
70 75 80	
ggg ctg gtc ttc gac gcc acc ggg atc acc acc ccg gag ggc ctg aag	345
Gly Leu Val Phe Asp Ala Thr Gly Ile Thr Thr Pro Glu Gly Leu Lys	
85 90 95 100	
ggg ctg tac gag ttc ttc acc cca ctg ctg cgc aac ctg ggt cac tgc	393
Gly Leu Tyr Glu Phe Phe Thr Pro Leu Leu Arg Asn Leu Gly His Cys	
105 110 115	
gcc cgc gtg gtg gtg gtc ggc acc acg ccc gac gcc gcc gcc ggc ccg	441
Ala Arg Val Val Val Val Gly Thr Thr Pro Asp Ala Ala Ala Gly Pro	
120 125 130	
cac gag cgg atc gcc cag cgc gcc ctg gag ggc ttc acc cgg tca ttg	489
His Glu Arg Ile Ala Gln Arg Ala Leu Glu Gly Phe Thr Arg Ser Leu	
135 140 145	
ggc aag gag ctg cgc aac ggc tcg acg gtg gcg ctg gtg tac ctg tcg	537
Gly Lys Glu Leu Arg Asn Gly Ser Thr Val Ala Leu Val Tyr Leu Ser	
150 155 160	
ccg gcc gcc aaa ccc gcc gcg acg ggc ctg gag tcg acc atg cgg ttc	585
Pro Ala Ala Lys Pro Ala Ala Thr Gly Leu Glu Ser Thr Met Arg Phe	
165 170 175 180	
atc ctg tcg gcc aag tcc gcc tac gtc gac ggc cag gtc ttc tac gtc	633
Ile Leu Ser Ala Lys Ser Ala Tyr Val Asp Gly Gln Val Phe Tyr Val	
185 190 195	
ggc gag gcc gac tcc acc ccc ccg gcg gac tgg gaa cgg ccg ctg gac	681
Gly Glu Ala Asp Ser Thr Pro Pro Ala Asp Trp Glu Arg Pro Leu Asp	
200 205 210	
ggc aag gtc gcc atc gtg acc ggt gcg gcc cgc gga atc ggc gcc acg	729
Gly Lys Val Ala Ile Val Thr Gly Ala Ala Arg Gly Ile Gly Ala Thr	
215 220 225	
atc gcc gag gtg ttc gcc cgc gac ggc gcc cgc gtg gtc gcg atc gac	777
Ile Ala Glu Val Phe Ala Arg Asp Gly Ala Arg Val Val Ala Ile Asp	
230 235 240	
gtg gaa tcg gcc gcc gag acg ctg gcc gag acg gcc agc cgg gtc ggc	825
Val Glu Ser Ala Ala Glu Thr Leu Ala Glu Thr Ala Ser Arg Val Gly	
245 250 255 260	
ggc acc gcg ctg tgg ctc gac gtc acc gcc ccc gac gcc gtc gac aag	873

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Gly Thr Ala Leu Trp Leu Asp Val Thr Ala Pro Asp Ala Val Asp Lys	
265 270 275	
atc acc gag cac ctg cgc gag cac cac ggc ggt cac gcc gac atc ctg	921
Ile Thr Glu His Leu Arg Glu His His Gly Gly His Ala Asp Ile Leu	
280 285 290	
gtc aac aac gcc ggg atc acc cgc gac aag ctg ctg gcc aac atg gac	969
Val Asn Asn Ala Gly Ile Thr Arg Asp Lys Leu Leu Ala Asn Met Asp	
295 300 305	
gac gcg cgc tgg gac gcc gtg ttg gcc gtg aat ctg ctt gcc cca ctt	1017
Asp Ala Arg Trp Asp Ala Val Leu Ala Val Asn Leu Leu Ala Pro Leu	
310 315 320	
cgc ctt acc gaa ggg ctg gtg ggc aac ggc agc atc ggc gaa ggc ggc	1065
Arg Leu Thr Glu Gly Leu Val Gly Asn Gly Ser Ile Gly Glu Gly Gly	
325 330 335 340	
cgc atc gtc ggc ctt tcg tcg atg gcc ggc atc gcg ggc aac cgc ggc	1113
Arg Ile Val Gly Leu Ser Ser Met Ala Gly Ile Ala Gly Asn Arg Gly	
345 350 355	
cag acc aac tac gcc acc acc aag gca ggc atg atc ggc ctc acc cag	1161
Gln Thr Asn Tyr Ala Thr Thr Lys Ala Gly Met Ile Gly Leu Thr Gln	
360 365 370	
gcg ctg gcg ccg gag ctc tac gac aag ggc atc acc atc aac gcc gtc	1209
Ala Leu Ala Pro Glu Leu Tyr Asp Lys Gly Ile Thr Ile Asn Ala Val	
375 380 385	
gcg ccg gga ttc atc gag acc cag atg acg gcc gcc atc ccg ctg gcc	1257
Ala Pro Gly Phe Ile Glu Thr Gln Met Thr Ala Ala Ile Pro Leu Ala	
390 395 400	
acc cgc gag gtg ggg cgc cgg atg aac tcg ctg ctg cag ggc ggc cag	1305
Thr Arg Glu Val Gly Arg Arg Met Asn Ser Leu Leu Gln Gly Gly Gln	
405 410 415 420	
ccg gtg gac gtc gcc gaa acc atc gcc tac ttc gcc agc ccg gcg tcg	1353
Pro Val Asp Val Ala Glu Thr Ile Ala Tyr Phe Ala Ser Pro Ala Ser	
425 430 435	
aac gcg gtg acc ggc aac gtc atc cgg gtc tgc ggc cag gcg atg ctg	1401
Asn Ala Val Thr Gly Asn Val Ile Arg Val Cys Gly Gln Ala Met Leu	
440 445 450	
ggg gca tga	1410
Gly Ala	

&lt;210&gt; 8

&lt;211&gt; 454

&lt;212&gt; PRT

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;400&gt; 8

Val Ala Pro Lys Val Ser Ser Asp Leu Phe Ser Gln Ile Val Asn Ser
1 5 10 15

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Gly Pro Gly Ser Phe Leu Ala Lys Gln Leu Gly Val Pro Gln Pro Glu  
 20 25 30

Thr Leu Arg Arg Tyr Arg Pro Gly Asp Pro Pro Leu Ala Gly Ser Leu  
 35 40 45

Leu Ile Gly Gly Glu Gly Arg Val Val Glu Pro Leu Arg Ala Ala Leu  
 50 55 60

Ala Lys Asp Tyr Asp Leu Val Gly Asn Asn Leu Gly Gly Arg Trp Ala  
 65 70 75 80

Asp Arg Phe Gly Gly Leu Val Phe Asp Ala Thr Gly Ile Thr Thr Pro  
 85 90 95

Glu Gly Leu Lys Gly Leu Tyr Glu Phe Phe Thr Pro Leu Leu Arg Asn  
 100 105 110

Leu Gly His Cys Ala Arg Val Val Val Gly Thr Thr Pro Asp Ala  
 115 120 125

Ala Ala Gly Pro His Glu Arg Ile Ala Gln Arg Ala Leu Glu Gly Phe  
 130 135 140

Thr Arg Ser Leu Gly Lys Glu Leu Arg Asn Gly Ser Thr Val Ala Leu  
 145 150 155 160

Val Tyr Leu Ser Pro Ala Ala Lys Pro Ala Ala Thr Gly Leu Glu Ser  
 165 170 175

Thr Met Arg Phe Ile Leu Ser Ala Lys Ser Ala Tyr Val Asp Gly Gln  
 180 185 190

Val Phe Tyr Val Gly Glu Ala Asp Ser Thr Pro Pro Ala Asp Trp Glu  
 195 200 205

Arg Pro Leu Asp Gly Lys Val Ala Ile Val Thr Gly Ala Ala Arg Gly  
 210 215 220

Ile Gly Ala Thr Ile Ala Glu Val Phe Ala Arg Asp Gly Ala Arg Val  
 225 230 235 240

Val Ala Ile Asp Val Glu Ser Ala Ala Glu Thr Leu Ala Glu Thr Ala  
 245 250 255

Ser Arg Val Gly Gly Thr Ala Leu Trp Leu Asp Val Thr Ala Pro Asp  
 260 265 270

Ala Val Asp Lys Ile Thr Glu His Leu Arg Glu His His Gly Gly His  
 275 280 285

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Ala Asp Ile Leu Val Asn Asn Ala Gly Ile Thr Arg Asp Lys Leu Leu  
290 295 300

Ala Asn Met Asp Asp Ala Arg Trp Asp Ala Val Leu Ala Val Asn Leu  
305 310 315 320

Leu Ala Pro Leu Arg Leu Thr Glu Gly Leu Val Gly Asn Gly Ser Ile  
325 330 335

Gly Glu Gly Gly Arg Ile Val Gly Leu Ser Ser Met Ala Gly Ile Ala  
340 345 350

Gly Asn Arg Gly Gln Thr Asn Tyr Ala Thr Thr Lys Ala Gly Met Ile  
355 360 365

Gly Leu Thr Gln Ala Leu Ala Pro Glu Leu Tyr Asp Lys Gly Ile Thr  
370 375 380

Ile Asn Ala Val Ala Pro Gly Phe Ile Glu Thr Gln Met Thr Ala Ala  
385 390 395 400

Ile Pro Leu Ala Thr Arg Glu Val Gly Arg Arg Met Asn Ser Leu Leu  
405 410 415

Gln Gly Gly Gln Pro Val Asp Val Ala Glu Thr Ile Ala Tyr Phe Ala  
420 425 430

Ser Pro Ala Ser Asn Ala Val Thr Gly Asn Val Ile Arg Val Cys Gly  
435 440 445

Gln Ala Met Leu Gly Ala  
450

<210> 9

<211> 625

<212> DNA

<213> mycobacterium avium paratuberculosis

<220>

<221> misc\_feature

<222> (592)..(592)

<223> "n"

<220>

<221> CDS

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&lt;222&gt; (179) .. (625)

&lt;223&gt;

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (619) .. (619)

&lt;223&gt; "n"

&lt;400&gt; 9

```

aattcgcgca taccgcgtcac tggtcacaac gccacatgct ggtaggctgt ggaatcgagg      60
gtcaatccgg atcggacccc aacgtcgact tgtgggcgcc aattcgcggg ttttcgcca      120
gcaagtcgac gttcggcgcg aatcggtgag gtgggcacag gtgaatgacg aagaggac      178
atg ctg gtc gcc acg gtg cgg gcg ttc atc gac cgc gag gtc aaa ccg      226
Met Leu Val Ala Thr Val Arg Ala Phe Ile Asp Arg Glu Val Lys Pro
1          5          10          15
acc gtg cgc gag gtg gag cac gcc gat gcc tat ccc gag gcg tgg atc      274
Thr Val Arg Glu Val Glu His Ala Asp Ala Tyr Pro Glu Ala Trp Ile
          20          25          30
gag cag atg aag cgg atc ggg atc tac ggg ctg gcg gtg ccc gag gaa      322
Glu Gln Met Lys Arg Ile Gly Ile Tyr Gly Leu Ala Val Pro Glu Glu
          35          40          45
tac ggt ggt tcg ccg gtg tcc atg ccg tgc tac gtg cgg gtc acc gag      370
Tyr Gly Gly Ser Pro Val Ser Met Pro Cys Tyr Val Arg Val Thr Glu
          50          55          60
cag ctg gcg cgc ggc tgg atg agc ctg gcc ggg gcg atg ggc ggg cac      418
Gln Leu Ala Arg Gly Trp Met Ser Leu Ala Gly Ala Met Gly Gly His
65          70          75          80
acc gtg gtg gcc aag ctg cta acg ctg ttc ggc acc gag gac cas aag      466
Thr Val Val Ala Lys Leu Leu Thr Leu Phe Gly Thr Glu Asp Xaa Lys
          85          90          95
cgg gcc tac ctg ccg cgg atg gcc agc ggc gaa atc cgg gcc acc atg      514
Arg Ala Tyr Leu Pro Arg Met Ala Ser Gly Glu Ile Arg Ala Thr Met
          100          105          110
gcg ttg acc gag ccc sgc ggc ggc tcg gac ctg cag aac atg tcg acc      562
Ala Leu Thr Glu Pro Xaa Gly Gly Ser Asp Leu Gln Asn Met Ser Thr
          115          120          125
acc gcg ctg ccc gac ccc gac tcc gac ggn ctg gtg gtc aac ggg gcc      610
Thr Ala Leu Pro Asp Pro Asp Ser Asp Gly Leu Val Val Asn Gly Ala
          130          135          140
aag acc tgn atc aac      625
Lys Thr Xaa Ile Asn
145

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&lt;210&gt; 10

&lt;211&gt; 149

<212> PRT

<213> mycobacterium avium paratuberculosis

<220>

<221> misc\_feature

<222> (95)..(95)

<223> The 'Xaa' at location 95 stands for Gln, or His.

<220>

<221> misc\_feature

<222> (118)..(118)

<223> The 'Xaa' at location 118 stands for Gly, or Arg.

<220>

<221> misc\_feature

<222> (147)..(147)

<223> The 'Xaa' at location 147 stands for a stop codon, Trp, or Cys.

<220>

<221> misc\_feature

<222> (592)..(592)

<223> "n"

<220>

<221> misc\_feature

<222> (619)..(619)

<223> "n"

<400> 10

Met	Leu	Val	Ala	Thr	Val	Arg	Ala	Phe	Ile	Asp	Arg	Glu	Val	Lys	Pro
1				5					10					15	

Thr	Val	Arg	Glu	Val	Glu	His	Ala	Asp	Ala	Tyr	Pro	Glu	Ala	Trp	Ile
		20						25					30		

Glu	Gln	Met	Lys	Arg	Ile	Gly	Ile	Tyr	Gly	Leu	Ala	Val	Pro	Glu	Glu
		35					40					45			

Tyr	Gly	Gly	Ser	Pro	Val	Ser	Met	Pro	Cys	Tyr	Val	Arg	Val	Thr	Glu
	50						55				60				

Gln	Leu	Ala	Arg	Gly	Trp	Met	Ser	Leu	Ala	Gly	Ala	Met	Gly	Gly	His
65					70					75					80



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Thr Val Val Ala Lys Leu Leu Thr Leu Phe Gly Thr Glu Asp Xaa Lys  
                   85                                  90                                  95

Arg Ala Tyr Leu Pro Arg Met Ala Ser Gly Glu Ile Arg Ala Thr Met  
                   100                                  105                                  110

Ala Leu Thr Glu Pro Xaa Gly Gly Ser Asp Leu Gln Asn Met Ser Thr  
                   115                                  120                                  125

Thr Ala Leu Pro Asp Pro Asp Ser Asp Gly Leu Val Val Asn Gly Ala  
                   130                                  135                                  140

Lys Thr Xaa Ile Asn  
 145

<210> 11

<211> 241

<212> DNA

<213> mycobacterium avium paratuberculosis

<220>

<221> CDS

<222> (147)..(239)

<223>

<400> 11  
 gtgggggcaa gcccaattacg ttcgcatcga cccggcacag gcggtcgctc acgtcatcaa 60  
 catgccgctc atccccgatg aggctcgaat gaccttgcta cgcaggcgct gaacgcacga 120  
 cgaaacggac cggaggtgaa agggac atg agc cac gcc gat caa ctc gct cgg 173  
                                   Met Ser His Ala Asp Gln Leu Ala Arg  
                                   1                                  5  
 acg cac ctg gcg ccc gat cct gcg gac ctg tcg cgc ctg gtc gcc ggc 221  
 Thr His Leu Ala Pro Asp Pro Ala Asp Leu Ser Arg Leu Val Ala Gly  
 10                                  15                                  20                                  25  
 acc cac cac gac ccg cac gg 241  
 Thr His His Asp Pro His  
                                   30

<210> 12

<211> 31

<212> PRT

<213> mycobacterium avium paratuberculosis

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&lt;400&gt; 12

Met Ser His Ala Asp Gln Leu Ala Arg Thr His Leu Ala Pro Asp Pro  
 1 5 10 15

Ala Asp Leu Ser Arg Leu Val Ala Gly Thr His His Asp Pro His  
 20 25 30

&lt;210&gt; 13

&lt;211&gt; 236

&lt;212&gt; DNA

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (8)..(214)

&lt;223&gt;

&lt;400&gt; 13

ggacacc aac gtg acc ggg gtg ttt ctc acc gcc cag gcg gcg gcc cgg 49  
 Asn Val Thr Gly Val Phe Leu Thr Ala Gln Ala Ala Ala Arg  
 1 5 10

gcg atg atg cgg cag ggc cgc ggc ggc gcc atc atc acc acc gcc tcg 97  
 Ala Met Met Arg Gln Gly Arg Gly Gly Ala Ile Ile Thr Thr Ala Ser  
 15 20 25 30

atg tcc ggg cac atc atc aac gtc ccg cag cag gtc ggc cac tac tgc 145  
 Met Ser Gly His Ile Ile Asn Val Pro Gln Gln Val Gly His Tyr Cys  
 35 40 45

gcc agc aag gcg gcc gtg atc cag ctg acc aag gcc atg gcc gtc gaa 193  
 Ala Ser Lys Ala Ala Val Ile Gln Leu Thr Lys Ala Met Ala Val Glu  
 50 55 60

ttc tgc agg atc cgt cga ctc tagactcgag caagcttatg ca 236  
 Phe Cys Arg Ile Arg Arg Leu  
 65

&lt;210&gt; 14

&lt;211&gt; 69

&lt;212&gt; PRT

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;400&gt; 14

Asn Val Thr Gly Val Phe Leu Thr Ala Gln Ala Ala Ala Arg Ala Met  
 1 5 10 15

Met Arg Gln Gly Arg Gly Gly Ala Ile Ile Thr Thr Ala Ser Met Ser  
20 25 30

Gly His Ile Ile Asn Val Pro Gln Gln Val Gly His Tyr Cys Ala Ser  
35 40 45

Lys Ala Ala Val Ile Gln Leu Thr Lys Ala Met Ala Val Glu Phe Cys  
50 55 60

Arg Ile Arg Arg Leu  
65

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<210> 15
<211> 419
<212> DNA
<213> mycobacterium avium paratuberculosis
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<220>  
<221>  misc_feature  
<222>  (331)..(331)  
<223>  "n"
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```
<220>  
<221> misc_feature  
<222> (398)..(398)  
<223> "n"
```

```
<220>
<221> CDS
<222> (25) .. (417)
<223>
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```

<400> 15
cgggccaccgc acccagggga ggcc atg act cac acc aag gcc ggt cgt gcc
Met Thr His Thr Lys Ala Gly Arg Ala
1 5

gcg tgg ccg gcc gcc tgc gcg gtc gtc ctg tcc gcc gcc gcg ctg ttg
Ala Trp Pro Ala Ala Cys Ala Val Val Leu Ser Ala Ala Ala Leu Leu
10 15 20 25

tgc gcg gca gcg gcc gcc gcg gac gaa gcc gat gac gcg ttc ctc gcc
Cys Ala Ala Ala Ala Ala Ala Asp Glu Ala Asp Asp Ala Phe Leu Ala
30 35 40

```

```

ggc ctg gcc aag ggc ggg atc acc atg ttc gac gac gac gac gcg atc      195
Gly Leu Ala Lys Gly Gly Ile Thr Met Phe Asp Asp Asp Asp Ala Ile
              45                      50                      55

gcc atg gcc cac agc gtg tgc tcg agc atc gac gcc aac ccc aac gtg      243
Ala Met Gly His Ser Val Cys Ser Ser Ile Asp Ala Asn Pro Asn Val
              60                      65                      70

tcg atg ctg gcg ctg cgg ctg acc aag caa acc ccg ttg acg ccg aag      291
Ser Met Leu Ala Leu Arg Leu Thr Lys Gln Thr Pro Leu Thr Pro Lys
              75                      80                      85

caa tcc gcc tac ttc atc ggt ctt tcg gtc gcc agc tac ntg ccc gca      339
Gln Ser Gly Tyr Phe Ile Gly Leu Ser Val Ala Ser Tyr Xaa Pro Ala
              90                      95                      100                      105

gta caa gga cga cgt cga ccc ctc gct ggg ctg gct gat ccc gcc gcc      387
Val Gln Gly Arg Arg Pro Leu Ala Gly Leu Ala Asp Pro Ala Ala
              110                      115                      120

gct gat gtg ang ttg ccg gcc ggc atc ggc gt      419
Ala Asp Val Xaa Leu Pro Ala Gly Ile Gly
              125                      130

```

<210> 16

<211> 131

<212> PRT

<213> mycobacterium avium paratuberculosis

<220>

<221> misc\_feature

<222> (103)..(103)

<223> The 'Xaa' at location 103 stands for Met, Val, or Leu.

<220>

<221> misc\_feature

<222> (125)..(125)

<223> The 'Xaa' at location 125 stands for Lys, Arg, Thr, or Met.

<220>

<221> misc\_feature

<222> (331)..(331)

<223> "n"

<220>

<221> misc\_feature

<222> (398)..(398)

<223> "n"

&lt;400&gt; 16

Met Thr His Thr Lys Ala Gly Arg Ala Ala Trp Pro Ala Ala Cys Ala  
 1 5 10 15

Val Val Leu Ser Ala Ala Ala Leu Leu Cys Ala Ala Ala Ala Ala Ala  
 20 25 30

Asp Glu Ala Asp Asp Ala Phe Leu Ala Gly Leu Ala Lys Gly Gly Ile  
 35 40 45

Thr Met Phe Asp Asp Asp Asp Ala Ile Ala Met Gly His Ser Val Cys  
 50 55 60

Ser Ser Ile Asp Ala Asn Pro Asn Val Ser Met Leu Ala Leu Arg Leu  
 65 70 75 80

Thr Lys Gln Thr Pro Leu Thr Pro Lys Gln Ser Gly Tyr Phe Ile Gly  
 85 90 95

Leu Ser Val Ala Ser Tyr Xaa Pro Ala Val Gln Gly Arg Arg Arg Pro  
 100 105 110

Leu Ala Gly Leu Ala Asp Pro Ala Ala Ala Asp Val Xaa Leu Pro Ala  
 115 120 125

Gly Ile Gly  
 130

&lt;210&gt; 17

&lt;211&gt; 392

&lt;212&gt; DNA

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (94)..(390)

&lt;223&gt;

&lt;400&gt; 17

cggcgtagca tcgtcaagtc gttgcccgcg ctgatgccgg agcggcagta aggagttcgg 60

ctggtgcaaa aacgcttgcc cacagtcggt ttg gtg ctg acg gcc gtt gtc gcc 114  
 Val Leu Thr Ala Val Val Ala  
 1 5

ggt atc gcc ggg tgc agc gcg gcg cag acc gtg ccg cgc aag gcc gcc 162  
 Gly Ile Ala Gly Cys Ser Ala Ala Gln Thr Val Pro Arg Lys Ala Ala  
 10 15 20

cgg ctg acc atc gac ggt gcc acc cac acg acc cgc ccg ccg tcc tgc 210  
 Arg Leu Thr Ile Asp Gly Ala Thr His Thr Thr Arg Pro Pro Ser Cys  
 25 30 35  
 cgg cag gac cag atg tat cgg acc atc aac atc ccc gac cac gac ggt 258  
 Arg Gln Asp Gln Met Tyr Arg Thr Ile Asn Ile Pro Asp His Asp Gly  
 40 45 50 55  
 gga gtc gaa gcg gtg gtg ctg ctc agc ggt tac cgg gtg atg ccg cag 306  
 Gly Val Glu Ala Val Val Leu Leu Ser Gly Tyr Arg Val Met Pro Gln  
 60 65 70  
 tgg gtg aag atc cgg aac gtc gac ggc ttc acc ggc agt cta ctg gcc 354  
 Trp Val Lys Ile Arg Asn Val Asp Gly Phe Thr Gly Ser Leu Leu Ala  
 75 80 85  
 asg gcg gag tgg gcg acg cgc acg tcg atc tca cma at 392  
 Xaa Ala Trp Ala Thr Arg Thr Ser Ile Ser Xaa  
 90 95

<210> 18

<211> 99

<212> PRT

<213> mycobacterium avium paratuberculosis

<220>

<221> misc\_feature

<222> (88)..(88)

<223> The 'Xaa' at location 88 stands for Arg, or Thr.

<220>

<221> misc\_feature

<222> (99)..(99)

<223> The 'Xaa' at location 99 stands for Gln, or Pro.

<400> 18

Val Leu Thr Ala Val Val Ala Gly Ile Ala Gly Cys Ser Ala Ala Gln  
1 5 10 15

Thr Val Pro Arg Lys Ala Ala Arg Leu Thr Ile Asp Gly Ala Thr His  
20 25 30

Thr Thr Arg Pro Pro Ser Cys Arg Gln Asp Gln Met Tyr Arg Thr Ile  
35 40 45

Asn Ile Pro Asp His Asp Gly Gly Val Glu Ala Val Val Leu Leu Ser  
50 55 60

Gly Tyr Arg Val Met Pro Gln Trp Val Lys Ile Arg Asn Val Asp Gly  
65 70 75 80

Phe Thr Gly Ser Leu Leu Ala Xaa Ala Glu Trp Ala Thr Arg Thr Ser  
                   85                                  90                                  95

Ile Ser Xaa

<210> 19

<211> 1884

<212> DNA

<213> mycobacterium avium paratuberculosis

<220>

<221> CDS

<222> (13)..(1884)

<223>

<400> 19

taaccaggag ca atg gct cgt gcg gtc ggt atc gac ctc ggg acc acc aac	51
Met Ala Arg Ala Val Gly Ile Asp Leu Gly Thr Thr Asn	
1                                  5                                  10	
tcc gtc gtc gca gtc ctc gag ggc ggt gac ccc gtc gtc gtc gcc aac	99
Ser Val Val Ala Val Leu Glu Gly Gly Asp Pro Val Val Val Ala Asn	
15                                  20                                  25	
tcc gag ggc tcg cgg acc acc ccg tcc atc gtc gcg ttc gcc cgc aac	147
Ser Glu Gly Ser Arg Thr Thr Pro Ser Ile Val Ala Phe Ala Arg Asn	
30                                  35                                  40                                  45	
ggc gag gtg ctc gtc ggc cag ccc gcc aag aac cag gcg gtg acc aac	195
Gly Glu Val Leu Val Gly Gln Pro Ala Lys Asn Gln Ala Val Thr Asn	
50                                  55                                  60	
gtc gac cgc acc atc cgt tcg gtc aag cgg cac atg ggc acc gac tgg	243
Val Asp Arg Thr Ile Arg Ser Val Lys Arg His Met Gly Thr Asp Trp	
65                                  70                                  75	
tcc atc gag atc gac ggc aag aaa tac acc gct cag gag atc agc gcc	291
Ser Ile Glu Ile Asp Gly Lys Lys Tyr Thr Ala Gln Glu Ile Ser Ala	
80                                  85                                  90	
cgc gtg ctg atg aag ctc aag cgc gac gcc gag gcc tat ctg ggt gag	339
Arg Val Leu Met Lys Leu Lys Arg Asp Ala Glu Ala Tyr Leu Gly Glu	
95                                  100                                  105	
gac atc acc gac gcg gtc atc acc gta ccg gcg tac ttc aac gac gcc	387
Asp Ile Thr Asp Ala Val Ile Thr Val Pro Ala Tyr Phe Asn Asp Ala	
110                                  115                                  120                                  125	
cag cgt cag gcg acc aag gaa gcc ggc cag atc gcc ggc ctc aac gtg	435
Gln Arg Gln Ala Thr Lys Glu Ala Gly Gln Ile Ala Gly Leu Asn Val	
130                                  135                                  140	
ctg cgc atc gtc aac gag ccg acc gcg gcc gcg ctg gcc tac ggc ctg	483

Leu Arg Ile Val Asn Glu Pro Thr Ala Ala Ala Leu Ala Tyr Gly Leu	
145 150 155	
gac aag ggc gag aag gag cag acc atc ctg gtc ttc gac ctc ggc ggc	531
Asp Lys Gly Glu Lys Glu Gln Thr Ile Leu Val Phe Asp Leu Gly Gly	
160 165 170	
ggc acg ttc gac gtt tcg ctg ctc gag atc ggc gag ggt gtg gtc gag	579
Gly Thr Phe Asp Val Ser Leu Leu Glu Ile Gly Glu Gly Val Val Glu	
175 180 185	
gtc cgc gcc acc agc ggt gac aac caa ctc ggt ggc gac gac tgg gac	627
Val Arg Ala Thr Ser Gly Asp Asn Gln Leu Gly Gly Asp Asp Trp Asp	
190 195 200 205	
gac cgg atc gtc aac tgg ctg gtc gac aag ttc aag ggc acc agc ggc	675
Asp Arg Ile Val Asn Trp Leu Val Asp Lys Phe Lys Gly Thr Ser Gly	
210 215 220	
atc gac ctg acc aag gac aag atg gcc atg cag cgg ctg cgt gag gcc	723
Ile Asp Leu Thr Lys Asp Lys Met Ala Met Gln Arg Leu Arg Glu Ala	
225 230 235	
gcc gag aag gcc aag atc gag ttg tcc agc tcg cag agc acc tcg atc	771
Ala Glu Lys Ala Lys Ile Glu Leu Ser Ser Ser Gln Ser Thr Ser Ile	
240 245 250	
aac ctg ccc tac atc acc gtc gac gcg gac aag aac ccg ctg ttc ctc	819
Asn Leu Pro Tyr Ile Thr Val Asp Ala Asp Lys Asn Pro Leu Phe Leu	
255 260 265	
gac gag cag ctg acc cgc gcc gaa ttc cag cgc atc acc cag gat ctg	867
Asp Glu Gln Leu Thr Arg Ala Glu Phe Gln Arg Ile Thr Gln Asp Leu	
270 275 280 285	
ctg gac cgc acc cgt cag ccg ttc aag tcg gtg atc gcc gac gcc ggc	915
Leu Asp Arg Thr Arg Gln Pro Phe Lys Ser Val Ile Ala Asp Ala Gly	
290 295 300	
atc tcg gtg tcc gac atc gac cac gtg gtg ctg gtg ggt ggt tcc acc	963
Ile Ser Val Ser Asp Ile Asp His Val Val Leu Val Gly Gly Ser Thr	
305 310 315	
cgg atg ccc gcg gtg acc gac ctg gtc aag gaa ctc acc ggc ggc aag	1011
Arg Met Pro Ala Val Thr Asp Leu Val Lys Glu Leu Thr Gly Gly Lys	
320 325 330	
gag ccc aac aag ggc gtc aac ccc gac gag gtt gtc gcg gtg ggt gcc	1059
Glu Pro Asn Lys Gly Val Asn Pro Asp Glu Val Val Ala Val Gly Ala	
335 340 345	
gcc ctg cag gcc ggt gtg ctt aag ggc gag gtg aaa gac gtt ctg ctg	1107
Ala Leu Gln Ala Gly Val Leu Lys Gly Glu Val Lys Asp Val Leu Leu	
350 355 360 365	
ctt gac gtt acg ccg ctg agc ctg ggt atc gag acc aag ggt ggc gtg	1155
Leu Asp Val Thr Pro Leu Ser Leu Gly Ile Glu Thr Lys Gly Gly Val	
370 375 380	
atg acc aag ctg atc gaa cgc aac acc acc atc ccg acc aag cgg tcc	1203
Met Thr Lys Leu Ile Glu Arg Asn Thr Thr Ile Pro Thr Lys Arg Ser	
385 390 395	
gag acg ttc acc acg gcc gac gac aac cag ccg tcg gtg cag atc cag	1251
Glu Thr Phe Thr Thr Ala Asp Asp Asn Gln Pro Ser Val Gln Ile Gln	
400 405 410	
gtg tat cag ggt gag cgc gaa atc gcc gcg cac aac aag ctg ctc ggc	1299



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Val	Tyr	Gln	Gly	Glu	Arg	Glu	Ile	Ala	Ala	His	Asn	Lys	Leu	Leu	Gly		
415						420					425						
tcc	ttc	gag	ctg	acc	gga	att	ccg	ccg	gcg	ccc	cgc	ggc	gtg	ccg	cag		1347
Ser	Phe	Glu	Leu	Thr	Gly	Ile	Pro	Pro	Ala	Pro	Arg	Gly	Val	Pro	Gln		
430					435					440					445		
atc	gag	gtc	acc	ttc	gac	atc	gac	gcc	aac	ggc	atc	gtg	cac	gtc	acc		1395
Ile	Glu	Val	Thr		Phe	Asp	Ile	Asp	Ala	Asn	Gly	Ile	Val	His	Val	Thr	
				450					455						460		
gcc	aag	gac	aag	ggc	acc	ggt	aag	gag	aac	acg	atc	aag	atc	cag	gag		1443
Ala	Lys	Asp	Lys	Gly	Thr	Gly	Lys	Glu	Asn	Thr	Ile	Lys	Ile	Gln	Glu		
			465					470						475			
ggc	tcc	ggc	ctg	tcc	aag	gag	gag	atc	gac	cgg	atg	atc	aag	gac	gcc		1491
Gly	Ser	Gly	Leu	Ser	Lys	Glu	Glu	Ile	Asp	Arg	Met	Ile	Lys	Asp	Ala		
		480					485					490					
gag	gcg	cac	gcc	gag	gag	gac	cgc	aag	agg	cgc	gag	gaa	gcc	gac	gtc		1539
Glu	Ala	His	Ala	Glu	Glu	Asp	Arg	Lys	Arg	Arg	Glu	Glu	Ala	Asp	Val		
	495					500					505						
cgc	aac	caa	gcg	gaa	tcg	ctt	gtc	tac	cag	acg	gag	aag	ttc	gtc	aag		1587
Arg	Asn	Gln	Ala	Glu	Ser	Leu	Val	Tyr	Gln	Thr	Glu	Lys	Phe	Val	Lys		
510					515					520					525		
gac	cag	cgc	gag	gcc	gag	ggc	ggc	tcg	aag	gtt	ccc	gag	gag	acg	ctg		1635
Asp	Gln	Arg	Glu	Ala	Glu	Gly	Gly	Ser	Lys	Val	Pro	Glu	Glu	Thr	Leu		
				530					535						540		
tcc	aag	gtc	gac	gcc	gcg	atc	gcc	gac	gcc	aag	acg	gcc	ctg	ggc	ggc		1683
Ser	Lys	Val	Asp	Ala	Ala	Ile	Ala	Asp	Ala	Lys	Thr	Ala	Leu	Gly	Gly		
			545					550					555				
acc	gac	atc	acc	gcg	atc	aag	tcg	gcg	atg	gag	aag	ctc	ggc	cag	gag		1731
Thr	Asp	Ile	Thr	Ala	Ile	Lys	Ser	Ala	Met	Glu	Lys	Leu	Gly	Gln	Glu		
		560					565					570					
tcg	caa	gcg	ctg	gga	cag	gca	atc	tac	gag	gcc	acc	cag	gcc	gag	tcc		1779
Ser	Gln	Ala	Leu	Gly	Gln	Ala	Ile	Tyr	Glu	Ala	Thr	Gln	Ala	Glu	Ser		
	575					580					585						
gcc	cag	gct	ggc	ggg	ccg	gac	ggt	gcc	gcg	gcc	ggc	ggc	ggg	tcc	gga		1827
Ala	Gln	Ala	Gly	Gly	Pro	Asp	Gly	Ala	Ala	Ala	Gly	Gly	Gly	Ser	Gly		
590					595				600						605		
tcc	gcc	gac	gat	gtt	gtg	gac	gcg	gag	gtg	gtc	gac	gat	gac	cgg	gag		1875
Ser	Ala	Asp	Asp	Val	Val	Asp	Ala	Glu	Val	Val	Asp	Asp	Asp	Arg	Glu		
				610					615					620			
tcc	aag	tga															1884
Ser	Lys																

&lt;210&gt; 20

&lt;211&gt; 623

&lt;212&gt; PRT

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;400&gt; 20

Met Ala Arg Ala Val Gly Ile Asp Leu Gly Thr Thr Asn Ser Val Val  
 1 5 10 15  
 Ala Val Leu Glu Gly Gly Asp Pro Val Val Val Ala Asn Ser Glu Gly  
 20 25 30  
 Ser Arg Thr Thr Pro Ser Ile Val Ala Phe Ala Arg Asn Gly Glu Val  
 35 40 45  
 Leu Val Gly Gln Pro Ala Lys Asn Gln Ala Val Thr Asn Val Asp Arg  
 50 55 60  
 Thr Ile Arg Ser Val Lys Arg His Met Gly Thr Asp Trp Ser Ile Glu  
 65 70 75 80  
 Ile Asp Gly Lys Lys Tyr Thr Ala Gln Glu Ile Ser Ala Arg Val Leu  
 85 90 95  
 Met Lys Leu Lys Arg Asp Ala Glu Ala Tyr Leu Gly Glu Asp Ile Thr  
 100 105 110  
 Asp Ala Val Ile Thr Val Pro Ala Tyr Phe Asn Asp Ala Gln Arg Gln  
 115 120 125  
 Ala Thr Lys Glu Ala Gly Gln Ile Ala Gly Leu Asn Val Leu Arg Ile  
 130 135 140  
 Val Asn Glu Pro Thr Ala Ala Ala Leu Ala Tyr Gly Leu Asp Lys Gly  
 145 150 155 160  
 Glu Lys Glu Gln Thr Ile Leu Val Phe Asp Leu Gly Gly Gly Thr Phe  
 165 170 175  
 Asp Val Ser Leu Leu Glu Ile Gly Glu Gly Val Val Glu Val Arg Ala  
 180 185 190  
 Thr Ser Gly Asp Asn Gln Leu Gly Gly Asp Asp Trp Asp Asp Arg Ile  
 195 200 205  
 Val Asn Trp Leu Val Asp Lys Phe Lys Gly Thr Ser Gly Ile Asp Leu  
 210 215 220  
 Thr Lys Asp Lys Met Ala Met Gln Arg Leu Arg Glu Ala Ala Glu Lys  
 225 230 235 240  
 Ala Lys Ile Glu Leu Ser Ser Ser Gln Ser Thr Ser Ile Asn Leu Pro  
 245 250 255  
 Tyr Ile Thr Val Asp Ala Asp Lys Asn Pro Leu Phe Leu Asp Glu Gln  
 260 265 270

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Leu Thr Arg Ala Glu Phe Gln Arg Ile Thr Gln Asp Leu Leu Asp Arg  
 275 280 285

Thr Arg Gln Pro Phe Lys Ser Val Ile Ala Asp Ala Gly Ile Ser Val  
 290 295 300

Ser Asp Ile Asp His Val Val Leu Val Gly Gly Ser Thr Arg Met Pro  
 305 310 315 320

Ala Val Thr Asp Leu Val Lys Glu Leu Thr Gly Gly Lys Glu Pro Asn  
 325 330 335

Lys Gly Val Asn Pro Asp Glu Val Val Ala Val Gly Ala Ala Leu Gln  
 340 345 350

Ala Gly Val Leu Lys Gly Glu Val Lys Asp Val Leu Leu Leu Asp Val  
 355 360 365

Thr Pro Leu Ser Leu Gly Ile Glu Thr Lys Gly Gly Val Met Thr Lys  
 370 375 380

Leu Ile Glu Arg Asn Thr Thr Ile Pro Thr Lys Arg Ser Glu Thr Phe  
 385 390 395 400

Thr Thr Ala Asp Asp Asn Gln Pro Ser Val Gln Ile Gln Val Tyr Gln  
 405 410 415

Gly Glu Arg Glu Ile Ala Ala His Asn Lys Leu Leu Gly Ser Phe Glu  
 420 425 430

Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val  
 435 440 445

Thr Phe Asp Ile Asp Ala Asn Gly Ile Val His Val Thr Ala Lys Asp  
 450 455 460

Lys Gly Thr Gly Lys Glu Asn Thr Ile Lys Ile Gln Glu Gly Ser Gly  
 465 470 475 480

Leu Ser Lys Glu Glu Ile Asp Arg Met Ile Lys Asp Ala Glu Ala His  
 485 490 495

Ala Glu Glu Asp Arg Lys Arg Arg Glu Glu Ala Asp Val Arg Asn Gln  
 500 505 510

Ala Glu Ser Leu Val Tyr Gln Thr Glu Lys Phe Val Lys Asp Gln Arg  
 515 520 525

Glu Ala Glu Gly Gly Ser Lys Val Pro Glu Glu Thr Leu Ser Lys Val  
 530 535 540

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Asp Ala Ala Ile Ala Asp Ala Lys Thr Ala Leu Gly Gly Thr Asp Ile  
545 550 555 560

Thr Ala Ile Lys Ser Ala Met Glu Lys Leu Gly Gln Glu Ser Gln Ala  
565 570 575

Leu Gly Gln Ala Ile Tyr Glu Ala Thr Gln Ala Glu Ser Ala Gln Ala  
580 585 590

Gly Gly Pro Asp Gly Ala Ala Ala Gly Gly Gly Ser Gly Ser Ala Asp  
595 600 605

Asp Val Val Asp Ala Glu Val Val Asp Asp Asp Arg Glu Ser Lys  
610 615 620

&lt;210&gt; 21

&lt;211&gt; 1701

&lt;212&gt; DNA

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (76)..(1701)

&lt;223&gt;

&lt;400&gt; 21

gcagcctggt cgtccgtcgc gggcactgca cccggccagg acgtgtcatc cccaatccgg 60

aggaatcact tcgca atg gcc aag aca att gcg tac gac gaa gag gcc cgt 111  
Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg  
1 5 10

cgc ggc ctc gag cgg ggg ctc aac gcc ctc gcc gac gcg gta aag gtc 159  
Arg Gly Leu Glu Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val  
15 20 25

acg ttg ggc ccc aag ggt cgc aac gtc gtc ctg gag aag aag tgg ggt 207  
Thr Leu Gly Pro Lys Gly Arg Asn Val Val Leu Glu Lys Lys Trp Gly  
30 35 40

gcc ccc acg atc acc aac gat ggt gtg tcc atc gcc aag gag atc gag 255  
Ala Pro Thr Ile Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu  
45 50 55 60

ctg gag gac ccg tac gag aag atc ggc gcc gag ctg gtc aag gaa gtc 303  
Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu Val  
65 70 75

gcc aag aag acc gac gac gtc gcc ggt gac ggc acg acg acg gcc acg 351  
Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr Ala Thr  
80 85 90

gtg ctc gcc cag gcg ttg gtc cgc gag ggc ctg cgc aac gtc gcg gcc 399

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Val	Leu	Ala	Gln	Ala	Leu	Val	Arg	Glu	Gly	Leu	Arg	Asn	Val	Ala	Ala		
	95						100					105					
ggc	gcc	aac	ccg	ctg	ggt	ctc	aag	cgc	ggc	atc	gag	aag	gcc	gtc	gag		447
Gly	Ala	Asn	Pro	Leu	Gly	Leu	Lys	Arg	Gly	Ile	Glu	Lys	Ala	Val	Glu		
	110					115					120						
aag	gtc	acc	gag	acc	ctg	ctc	aag	tcg	gcc	aag	gag	gtc	gag	acc	aag		495
Lys	Val	Thr	Glu	Thr		Leu	Lys	Ser	Ala	Lys	Glu	Val	Glu	Thr	Lys		
125					130				135						140		
gac	cag	atc	gct	gcc	acc	gcg	gcc	atc	tcc	gcg	ggc	gac	cag	tcg	atc		543
Asp	Gln	Ile	Ala	Ala	Thr	Ala	Ala	Ile	Ser	Ala	Gly	Asp	Gln	Ser	Ile		
			145						150						155		
ggc	gac	ctg	atc	gcc	gag	gcg	atg	gac	aag	gtc	ggc	aac	gag	ggc	gtc		591
Gly	Asp	Leu	Ile	Ala	Glu	Ala	Met	Asp	Lys	Val	Gly	Asn	Glu	Gly	Val		
		160						165					170				
atc	acc	gtc	gag	gag	tcc	aac	acc	ttc	ggc	ctg	cag	ctc	gag	ctc	acc		639
Ile	Thr	Val	Glu	Glu	Ser	Asn	Thr	Phe	Gly	Leu	Gln	Leu	Glu	Leu	Thr		
		175					180					185					
gag	ggt	atg	cgg	ttc	gac	aag	ggt	tac	atc	tcg	ggc	tac	ttc	gtc	acg		687
Glu	Gly	Met	Arg	Phe	Asp	Lys	Gly	Tyr	Ile	Ser	Gly	Tyr	Phe	Val	Thr		
	190					195					200						
gac	gcc	gag	cgt	cag	gaa	gcg	gtc	ctc	gag	gac	ccg	ttc	atc	ctg	ctg		735
Asp	Ala	Glu	Arg	Gln	Glu	Ala	Val	Leu	Glu	Asp	Pro	Phe	Ile	Leu	Leu		
205					210				215					220			
gtc	agc	tcc	aag	gtc	tcg	acc	gtc	aag	gac	ctg	ctg	ccg	ctg	ctg	gag		783
Val	Ser	Ser	Lys	Val	Ser	Thr	Val	Lys	Asp	Leu	Leu	Pro	Leu	Leu	Glu		
				225					230					235			
aag	gtc	atc	cag	gcc	ggc	aag	ccg	ctg	ctg	atc	atc	gcc	gag	gac	gtc		831
Lys	Val	Ile	Gln	Ala	Gly	Lys	Pro	Leu	Leu	Ile	Ile	Ala	Glu	Asp	Val		
			240				245						250				
gag	ggc	gag	gcc	ctg	tcc	acc	ctg	gtc	gtc	aac	aag	atc	cgc	ggc	acc		879
Glu	Gly	Glu	Ala	Leu	Ser	Thr	Leu	Val	Val	Asn	Lys	Ile	Arg	Gly	Thr		
		255					260					265					
ttc	aag	tcg	gtg	gcc	gtc	aag	gcg	ccc	ggc	ttc	ggc	gac	cgc	cgc	aag		927
Phe	Lys	Ser	Val	Ala	Val	Lys	Ala	Pro	Gly	Phe	Gly	Asp	Arg	Arg	Lys		
	270					275					280						
gcg	atg	ctt	cag	gac	atg	gcc	atc	ctc	acc	ggc	ggc	cag	gtc	atc	agc		975
Ala	Met	Leu	Gln	Asp	Met	Ala	Ile	Leu	Thr		Gly	Gly	Gln	Val	Ile	Ser	
285					290					295					300		
gaa	gag	gtc	ggc	ctg	tcg	ctg	gag	agc	gcc	gac	atc	tcg	ctg	ctc	ggt		1023
Glu	Glu	Val	Gly	Leu	Ser	Leu	Glu	Ser	Ala	Asp	Ile	Ser	Leu	Leu	Gly		
				305					310					315			
aag	gcc	cgc	aag	gtc	gtc	gtc	acc	aag	gac	gag	acc	acc	atc	gtc	gag		1071
Lys	Ala	Arg	Lys	Val	Val	Val	Thr	Lys	Asp	Glu	Thr	Thr	Ile	Val	Glu		
			320					325					330				
ggc	gcc	ggt	gac	tcc	gac	gcc	atc	gcc	ggc	cgg	gtg	gcc	cag	atc	cgc		1119
Gly	Ala	Gly	Asp	Ser	Asp	Ala	Ile	Ala	Gly	Arg	Val	Ala	Gln	Ile	Arg		
		335					340					345					
acc	gag	atc	gag	aac	agc	gac	tcc	gac	tac	gac	cgc	gag	aag	ctg	cag		1167
Thr	Glu	Ile	Glu	Asn	Ser	Asp	Ser	Asp	Tyr	Asp	Arg	Glu	Lys	Leu	Gln		
		350				355					360						
gag	cgg	ctg	gcc	aag	ctg	gcc	ggc	ggc	gtg	gcg	gtg	atc	aag	gcc	ggc		1215

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Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys Ala Gly	
365 370 375 380	
gcc gcg acc gag gtc gag ctc aag gag cgc aag cac cgc atc gag gac	1263
Ala Ala Thr Glu Val Glu Leu Lys Glu Arg Lys His Arg Ile Glu Asp	
385 390 395	
gcg gtc cgc aac gcc aag gcg gcc gtg gag gag ggc atc gtc gcc ggc	1311
Ala Val Arg Asn Ala Lys Ala Ala Val Glu Glu Gly Ile Val Ala Gly	
400 405 410	
ggc ggc gtc gcc ctg ctg cac gcg atc ccg gct ctg gac gag ctg aag	1359
Gly Gly Val Ala Leu Leu His Ala Ile Pro Ala Leu Asp Glu Leu Lys	
415 420 425	
ctc gag ggc gaa gag gcg acc ggc gcc aac atc gtc cgg gtg gcc ctc	1407
Leu Glu Gly Glu Glu Ala Thr Gly Ala Asn Ile Val Arg Val Ala Leu	
430 435 440	
gag gct ccg ctg aag cag atc gcc ttc aac ggt ggc ctg gag ccc ggc	1455
Glu Ala Pro Leu Lys Gln Ile Ala Phe Asn Gly Gly Leu Glu Pro Gly	
445 450 455 460	
gtg gtg gcc gag aag gtc cgc aac tcg ccc gcc ggt acc ggc ctc aac	1503
Val Val Ala Glu Lys Val Arg Asn Ser Pro Ala Gly Thr Gly Leu Asn	
465 470 475	
gcc gcc acc ggt gag tac gag gac ctg ctc aag gcc ggc att gcc gac	1551
Ala Ala Thr Gly Glu Tyr Glu Asp Leu Leu Lys Ala Gly Ile Ala Asp	
480 485 490	
ccg gtg aag gtc acc cgc tcg gcg ctg cag aac gcg gcg tcc atc gcg	1599
Pro Val Lys Val Thr Arg Ser Ala Leu Gln Asn Ala Ala Ser Ile Ala	
495 500 505	
ggg ctg ttc ctg acc acc gag gcg gtc gtc gcc gac aag ccg gag aag	1647
Gly Leu Phe Leu Thr Thr Glu Ala Val Val Ala Asp Lys Pro Glu Lys	
510 515 520	
gcg gcc gct ccc gcg ggc gac ccg acc ggc ggc atg ggc ggc atg gac	1695
Ala Ala Ala Pro Ala Gly Asp Pro Thr Gly Gly Met Gly Gly Met Asp	
525 530 535 540	
ttc tga	1701
Phe	

&lt;210&gt; 22

&lt;211&gt; 541

&lt;212&gt; PRT

&lt;213&gt; mycobacterium avium paratuberculosis

&lt;400&gt; 22

Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu Glu
1 5 10 15

Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu Gly Pro
20 25 30

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Lys Gly Arg Asn Val Val Leu Glu Lys Lys Trp Gly Ala Pro Thr Ile  
 35 40 45

Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu Leu Glu Asp Pro  
 50 55 60

Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu Val Ala Lys Lys Thr  
 65 70 75 80

Asp Asp Val Ala Gly Asp Gly Thr Thr Thr Ala Thr Val Leu Ala Gln  
 85 90 95

Ala Leu Val Arg Glu Gly Leu Arg Asn Val Ala Ala Gly Ala Asn Pro  
 100 105 110

Leu Gly Leu Lys Arg Gly Ile Glu Lys Ala Val Glu Lys Val Thr Glu  
 115 120 125

Thr Leu Leu Lys Ser Ala Lys Glu Val Glu Thr Lys Asp Gln Ile Ala  
 130 135 140

Ala Thr Ala Ala Ile Ser Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile  
 145 150 155 160

Ala Glu Ala Met Asp Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu  
 165 170 175

Glu Ser Asn Thr Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg  
 180 185 190

Phe Asp Lys Gly Tyr Ile Ser Gly Tyr Phe Val Thr Asp Ala Glu Arg  
 195 200 205

Gln Glu Ala Val Leu Glu Asp Pro Phe Ile Leu Leu Val Ser Ser Lys  
 210 215 220

Val Ser Thr Val Lys Asp Leu Leu Pro Leu Leu Glu Lys Val Ile Gln  
 225 230 235 240

Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu Ala  
 245 250 255

Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys Ser Val  
 260 265 270

Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala Met Leu Gln  
 275 280 285

Asp Met Ala Ile Leu Thr Gly Gly Gln Val Ile Ser Glu Glu Val Gly  
 290 295 300

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Leu Ser Leu Glu Ser Ala Asp Ile Ser Leu Leu Gly Lys Ala Arg Lys  
 305 310 315 320

Val Val Val Thr Lys Asp Glu Thr Thr Ile Val Glu Gly Ala Gly Asp  
 325 330 335

Ser Asp Ala Ile Ala Gly Arg Val Ala Gln Ile Arg Thr Glu Ile Glu  
 340 345 350

Asn Ser Asp Ser Asp Tyr Asp Arg Glu Lys Leu Gln Glu Arg Leu Ala  
 355 360 365

Lys Leu Ala Gly Gly Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu  
 370 375 380

Val Glu Leu Lys Glu Arg Lys His Arg Ile Glu Asp Ala Val Arg Asn  
 385 390 395 400

Ala Lys Ala Ala Val Glu Glu Gly Ile Val Ala Gly Gly Gly Val Ala  
 405 410 415

Leu Leu His Ala Ile Pro Ala Leu Asp Glu Leu Lys Leu Glu Gly Glu  
 420 425 430

Glu Ala Thr Gly Ala Asn Ile Val Arg Val Ala Leu Glu Ala Pro Leu  
 435 440 445

Lys Gln Ile Ala Phe Asn Gly Gly Leu Glu Pro Gly Val Val Ala Glu  
 450 455 460

Lys Val Arg Asn Ser Pro Ala Gly Thr Gly Leu Asn Ala Ala Thr Gly  
 465 470 475 480

Glu Tyr Glu Asp Leu Leu Lys Ala Gly Ile Ala Asp Pro Val Lys Val  
 485 490 495

Thr Arg Ser Ala Leu Gln Asn Ala Ala Ser Ile Ala Gly Leu Phe Leu  
 500 505 510

Thr Thr Glu Ala Val Val Ala Asp Lys Pro Glu Lys Ala Ala Ala Pro  
 515 520 525

Ala Gly Asp Pro Thr Gly Gly Met Gly Gly Met Asp Phe  
 530 535 540



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ity model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA,  
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*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: **DIAGNOSTICS AND VACCINES FOR MYCOBACTERIUM PARATUBERCULOSIS INFECTIONS**

(57) Abstract: The present invention relates to nucleic acid sequences encoding *Mycobacterium avium* subspecies *paratuberculosis* proteins, to parts of such nucleic acid sequences that encode an immunogenic fragment of such proteins, to DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof. The invention also relates to *Mycobacterium avium* subspecies *paratuberculosis* proteins and immunogenic parts thereof encoded by such sequences. Furthermore, the present invention relates to vaccines comprising such nucleic acid sequences and parts thereof, DNA fragments, recombinant DNA molecules, live recombinant carriers and host cells comprising such nucleic acid sequences or such parts thereof, proteins or immunogenic parts thereof and antibodies against such proteins or immunogenic parts thereof. Also, the invention relates to the use of said proteins in vaccines and for the manufacture of vaccines. Moreover, the invention relates to the use of said nucleic acid sequences, proteins or antibodies for diagnostic or vaccination purposes. Also, the invention relates to methods for the preparation of such vaccines. Finally the invention relates to diagnostic kits comprising such nucleic acids, proteins or antibodies against such proteins.

WO 03/058248 A3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL 03/00020

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/569 C12N5/10 C07K14/35 C12N15/31 C07K16/12  
C12Q1/68 C12Q1/02 A61K39/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols),

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used),

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 16628 A (INNOGENETICS NV) 1 October 1992 (1992-10-01) cited in the application abstract page 5, last paragraph -page 6, line 16 page 18, line 19-26 page 20, line 29 -page 21, line 13 page 22 -page 27, line 29 page 58, line 31 -page 59, line 30; table 3 claims 1-28	1,12-16, 27-37
X	EP 0 288 306 A (MCFADDEN JOHN JO ;TAYLOR JOHN HERMON (GB)) 26 October 1988 (1988-10-26) cited in the application abstract column 7, line 51 -column 8, line 11 -/-	1,12-16, 27-37

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

25 June 2003

Date of mailing of the international search report

18.09.03

Name and mailing address of the ISA

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Authorized officer

Dumont, E

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 03/00020

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KESEL DE M ET AL: "CLONING AND EXPRESSION OF PORTIONS OF THE 34-KILODALTON- PROTEIN GENE OF MYCOBACTERIUM PARATUBERCULOSIS: ITS APPLICATION TO SEROLOGICAL ANALYSIS OF JOHNE'S DISEASE" JOURNAL OF CLINICAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 31, no. 4, April 1993 (1993-04), pages 947-954, XP000978898 ISSN: 0095-1137 cited in the application the whole document	1,12-16, 27-37
X	EL-ZAATARI FOUAD A K ET AL: "Characterization of a specific Mycobacterium paratuberculosis recombinant clone expressing 35,000-molecular-weight antigen and reactivity with sera from animals with clinical and subclinical Johne's disease." JOURNAL OF CLINICAL MICROBIOLOGY, vol. 35, no. 7, 1997, pages 1794-1799, XP002201774 ISSN: 0095-1137 the whole document	1,12-16, 27-37

# INTERNATIONAL SEARCH REPORT

ational application No.  
PCT/NL 03/00020

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1, 16 (complete), 12-15, 27-37 (partial)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1, 16 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 5, encoding an amino acid sequence according to SEQ ID NO: 6.

2. Claims: 2, 17 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 3, encoding an amino acid sequence according to SEQ ID NO: 4.

3. Claims: 3, 18 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 1, encoding an amino acid sequence according to SEQ ID NO: 2.

4. Claims: 4, 19 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 7, encoding an amino acid sequence according to SEQ ID NO: 8.

5. Claims: 5, 20 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 9, encoding an amino acid sequence according to SEQ ID NO: 10.

6. Claims: 6, 21 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 11, encoding an amino acid sequence according to SEQ ID NO: 12.

7. Claims: 7, 22 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according to SEQ ID NO: 13, encoding an amino acid sequence according to SEQ ID NO: 14.

8. Claims: 8, 23 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

to SEQ ID NO: 15, encoding an amino acid sequence according  
to SEQ ID NO: 16.

9. Claims: 9, 24 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence according  
to SEQ ID NO: 17, encoding an amino acid sequence according  
to SEQ ID NO: 18.

10. Claims: 10, 25 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence encoding  
a 60 kD Mycobacterium avium subspecies paratuberculosis  
protein having a pI of 5,60-6,15.

11. Claims: 11, 26 (complete), 12-15, 27-37 (partial)

subject-matter relating to a nucleic acid sequence encoding  
a 33 kD Mycobacterium avium subspecies paratuberculosis  
protein having a pI of 4.20-4.75.

# INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/NL 03/00020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9216628	A	01-10-1992	AT 197069 T 15-11-2000
		AU 657743 B2 23-03-1995	
		AU 1441092 A 21-10-1992	
		DE 69231519 D1 23-11-2000	
		DE 69231519 T2 31-05-2001	
		WO 9216628 A1 01-10-1992	
		EP 0577666 A1 12-01-1994	
		ES 2152928 T3 16-02-2001	
		US 6387372 B1 14-05-2002	
EP 0288306	A	26-10-1988	AT 131533 T 15-12-1995
		AU 624574 B2 18-06-1992	
		AU 1628688 A 02-12-1988	
		CA 1340172 C 08-12-1998	
		DE 3854767 D1 25-01-1996	
		DE 3854767 T2 05-09-1996	
		DK 524289 A 22-12-1989	
		EP 0288306 A1 26-10-1988	
		EP 0356450 A1 07-03-1990	
		WO 8808456 A1 03-11-1988	
		JP 3503837 T 29-08-1991	
		NO 885756 A 23-02-1989	
		NZ 224351 A 21-12-1990	
		US 5225324 A 06-07-1993	

# PATENT COOPERATION TREATY

# PCT

REC'D 05 JAN 2004

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## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

(Rationalised Report according to the Notice of the President of the EPO published in the OJ11/2001)

Applicant's or agent's file reference <b>P54977PC00</b>	<div style="display: flex; justify-content: space-between;"> <div><b>FOR FURTHER ACTION</b></div> <div>See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)</div> </div>	
International application No. <b>PCT/NL 03/ 00020</b>	International filing date (day/month/year) <b>13/01/2003</b>	Priority date (day/month/year) <b>11/01/2002</b>
International Patent Classification (IPC) or national classification and IPC <b>G01N33/569</b>		
Applicant <b>ID-LELYSTAD, INSTITUUT VOOR DIERHOUDERIJ ...et al.</b>		

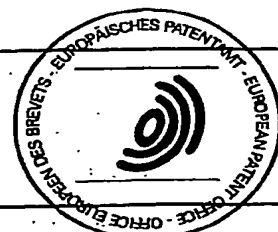
1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This **REPORT** consists of a total of 2 sheets, including this cover sheet.  
  
☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of \_\_\_\_\_ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand <b>08/08/2003</b>	Date of completion of this report <b>23/12/2003</b>
Name and mailing address of the IPEA/  <div style="display: inline-block; vertical-align: middle;"> European Patent Office  D-80298 Munich  Tel. (+49-89) 2399-0, Tx: 523656 epmu d  Fax: (+49-89) 2399-4465 </div>	Authorized officer <b>GUGERELL C</b>  Tel. (+49-89) 2399 2828





**I. Basis of the report**

The basis of this international preliminary examination is the application as originally filed.

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The question of whether the claimed invention appears to be novel, to involve an inventive step, or to be industrially applicable has not been the subject of the international preliminary examination in respect of the claims which have not been searched (Article 17(2)(a) or (3) and Rule 66.1(e) PCT); see also international search report).

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability**

To the extent that the international preliminary examination has been carried out (see item III above), the following is pointed out:

In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims, which have been the subject of an international search report, does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claim references).

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